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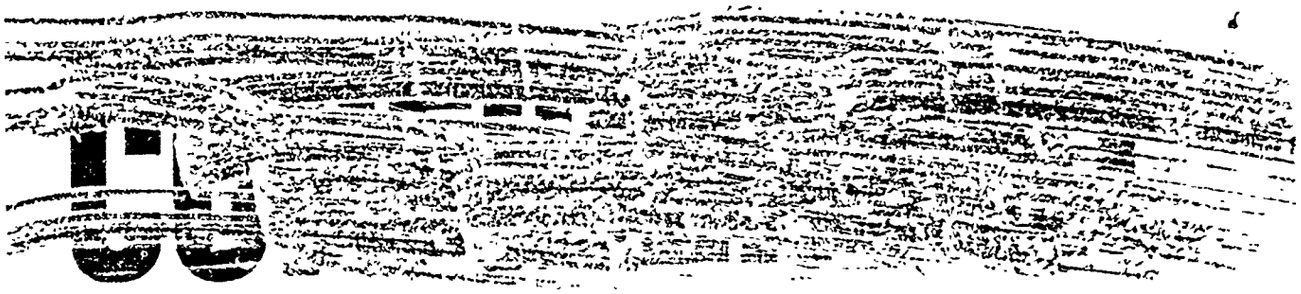

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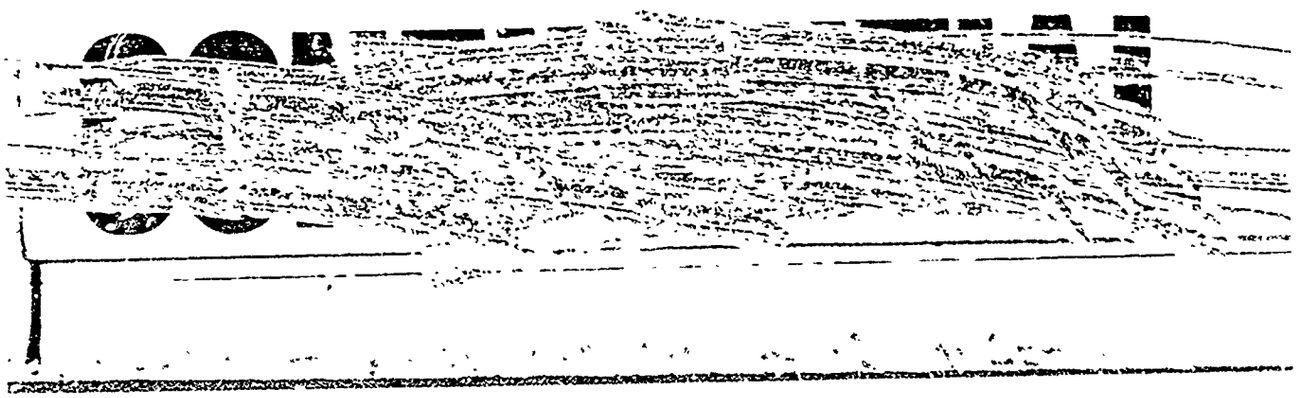

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TECHNICAL REPORT
BWL 23

SUMMATION OF SCREENING INVESTIGATIONS OF ACTINO-BACILLUS MALLEI AND PSEUDOMONAS PSEUDOMALLEI (U)

Benjamin J. Wilson
James E. Ogg
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November 1959

U.S. ARMY CHEMICAL CORPS
BIOLOGICAL WARFARE LABORATORIES
FORT DETRICK

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U. S. ARMY BIOLOGICAL WARFARE LABORATORIES
Fort Detrick, Frederick, Maryland

BWL Technical Report 23

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Special Operations Division
DEPUTY COMMANDER FOR SCIENTIFIC ACTIVITIES

Project 4-04-14-004

November 1959

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CMLRD-BL-12-3
(21 Dec 57) Jan 7 1965

(U) FOREWORD

(U) The screening investigations of the two bacterial species described in this report were performed under Special BW Operations Project 4-04-14-004. From 1952 to 1954 investigations were concerned principally with Actinobacillus mallei (Malleomyces mallei). In July 1953, virulent strains of Pseudomonas pseudomallei (Malleomyces pseudomallei) became available, and the investigation of A. mallei was gradually phased out. After a period of inactivity covering most of 1955 and 1956 because of higher priority given other tasks and lack of technical personnel, work was resumed on P. pseudomallei.

(U) Appreciation is expressed to members of Technical Evaluation Division who assessed the aerosol properties of several agent preparations.

(U) In addition to the authors, personnel participating at various times in the investigations include: George W. Schmersahl, John Thiel, John L. Bradshaw, William C. Wheeler, William S. Woodrow, Ralph J. Groomes, Harold L. Baier, PFC Seymour S. Shankman, Lt. Carl Bruch, CmlC, USA, and Lt. Col. J. R. MacNamee, VC, USA. The contributions of these persons are gratefully acknowledged. Appreciation also is expressed to Mr. James A. Kime for technical review and assistance in preparing this report.

(U) ABSTRACT

(U) Actinobacillus (Malleomyces) mallei and Pseudomonas (Malleomyces) pseudomallei were investigated for their potential as biological warfare agents. The preliminary studies described cover a range of topics including culture procedures, animal virulence, aerosol stability and infectivity, storage stability, and therapy of experimental infections.

(C) DIGEST

(C) Several strains of Actinobacillus mallei and Pseudomonas pseudomallei were collected and screened with respect to virulence for laboratory animals. One virulent strain of each species was selected for a general study that included methods of culture, aerosol stability, storage stability, drying stability, and host susceptibility. Data reported provide a basis for predicting the potential value of these agents for special operations.

(C) Virulent strains of A. mallei apparently have a high degree of biological effectiveness against man. High concentrations of the organism may be produced in simple aerated medium but research on this agent was discontinued because of the poor storage stability and the gradual or abrupt decrease in virulence.

(U) Although human infections with P. pseudomallei have been infrequently reported, those cases clinically recognized usually have been fatal. Preliminary clinical and laboratory data indicate that the newer "broad spectrum" antibiotics may reduce the mortality rate considerably.

(C) P. pseudomallei may be produced in high concentrations by using simple medium and facilities for aerating cultures. Fresh suspensions possess adequate aerosol stability, marked infectivity for susceptible animals, and storage stability which compare favorably with that of other vegetative bacteria studied at BW Labs. Agent suspensions may be dried so that temporary loss of virulence is avoided.

(C) To determine with greater reliability the disease-producing potential of P. pseudomallei in man, this agent should be assessed by research methods that allow an indirect correlation of laboratory animal infectivity and lethality with those observed in human populations.

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I. (C) INTRODUCTION

(U) The importance of Actinobacillus mallei (Malleomyces mallei) and Pseudomonas pseudomallei (Malleomyces pseudomallei) as causative agents of two lethal diseases in both animals and man has been recognized for many years. Therefore, the selection of these two bacterial species for screening as potential biological warfare agents appears logical.

(C) The first concerted study of these organisms at Fort Detrick was made by Miller et al during 1944 and 1945; the findings are recorded in Special Report 53.[1] The favorable results stimulated interest, and research was initiated on these bacterial species for special BW operations. The investigations were carried out in accordance with a research guide formulated for screening, adapting, and/or developing these organisms to meet operational requirements.

(U) Because of the high animal infectivity and human mortality rates reported for both species of bacteria, all work with the living organisms was performed with the Class III cabinets in the laboratory section of Building 459.

[1] See Literature Cited.

II. (C) ACTINOBACILLUS MALLEI AND GLANDERS

A. (C) DISEASE HISTORY

(U) Glanders has been known since antiquity, primarily as a natural disease of equine animals. In horses and asses involvement of the superficial lymphatics gives rise to a condition known as "farcy" in which the swollen lymph vessels and glands are discernible beneath the skin as "farcy pipes" and "farcy buds." In those acute or chronic cases involving the lungs, the term "glanders"* is applied.[2]

(U) Other animals that occasionally become infected with A. mallei include guinea pigs, cats, dogs, ferrets, moles, field mice, goats, sheep, hogs, and rabbits. Man appears to have been an incidental host in spite of the fact that the organism has exhibited a marked contagiousness among horses. Most cases of human disease apparently resulted from direct contact with infected animals, although man-to-man transmission has been documented.[3] This organism, like Pasteurella tularensis, is very infectious for man. Wilson and Miles[2] were most emphatic about infectivity: "Indeed probably no organism with the possible exception of Br. tularensis is so dangerous to work with as the glanders bacillus."

B. (C) INCIDENCE OF THE DISEASE

(C) The disease was fairly widespread in equine animals on the European continent, in Great Britain, and in North America prior to the turn of the century. It has also been found in Asia and Africa. The recognition of the contagiousness of the disease and the specific causative agent resulted in the imposition of certain preventive measures[2] which have had much to do with its suppression or low incidence in most countries for many years. The replacement of the equine beasts of burden with mechanized equipment in many areas of the world will undoubtedly tend to maintain a low incidence of the disease in the future.

C. (C) DISEASE IN MAN

(C) The over-all mortality rate of glanders as it was formerly noted in man was quite high, 90 to 100 per cent.[3]

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* In this report the word "glanders" will apply to all manifestations of infection with Actinobacillus mallei.

(C) Two distinct clinical forms have been recognized, acute and chronic. Acute glanders has a sudden onset and may appear as a bronchopneumonia or lobar pneumonia followed by development of pyemia with the production of characteristic abscesses in many organs including lymph nodes, liver, lungs, and spleen. Involvement of the upper respiratory tract in man may result in a purulent, infectious nasal discharge similar to that seen in other susceptible animals. Suppurative skin lesions are also frequently noted. Before the advent of the sulfonamides, death would almost always result within seven to ten days after disease onset.2/ Chronic manifestations of the disease have been known to persist for years with the appearance of granulomatous or suppurative skin and joint lesions.3,4/

(U) Because of the protean manifestations of the disease and its resemblance to a variety of more commonly noted afflictions of man, the correct diagnosis has often been missed until the patient has either become a potential source of infection or has died. Exudates, excretions, and the blood may contain glanders bacilli capable of re-establishing infection in contacts. The most closely related disease is melioidosis (discussed later) in which the pathological picture is quite similar and the causative organism cannot be readily differentiated without extensive and carefully controlled bacteriological tests.

(C) The fundamental factors on which the destructive influences of this organism depend reside in its notorious ability to parasitize the host with the production of a typical tissue necrosis and general toxemia. The well-developed lesions found in various organs usually exhibit a characteristic caseous necrosis which in some respects resembles that resulting from tuberculosis. The pathology of the human disease is well described by Bernstein and Carling.4/

(C) The incubation period of glanders ranges from a few hours to several weeks.2/ In those cases resulting from laboratory accidents at Fort Detrick during World War II the period varied from 10 to 14 days.1,5/ These infections probably resulted from inhalation of a small number of organisms.

D. (C) ROUTES OF INFECTION

(U) The most frequent avenue of infection postulated for human glanders has been the alimentary route. Although this may have been true in many instances, data presented in this and other reports suggest that man, as well as the lower animals, may readily acquire the disease by inhaling airborne particles or droplets containing the bacilli. There is also evidence that the disease may be induced by bacteria from contaminated fomites which enter the body through cuts or abrasions.3/

E. (U) PROPHYLAXIS

(U) No satisfactory prophylactic or immunological procedure has been devised to protect against glanders in man. The following vaccines have been reported ineffective in preventing reinfection in the horse: (a) living bacilli in minute doses, (b) killed organisms, and (c) mallein (spent culture concentrate).[2]

F. (U) TREATMENT

(U) Before the advent of the sulfonamides and antibiotics there was no specific treatment known for this disease. The six cases that occurred at Fort Detrick during World War II were successfully treated with sulfadiazine.[1,5] Reports emanating from the Defence Research Kingston Laboratories of Canada[8] indicate that sulfadiazine is also effective in preventing death in hamsters exposed to an otherwise lethal number of organisms as an aerosol. Many of the antibiotics have not been tried clinically against glanders because of its rarity in most countries. Because of the in vitro susceptibility of glanders bacilli to various antibiotics, however, (vide infra) there is reason to believe that they also could be employed successfully in therapy.

G. (U) CAUSATIVE ORGANISM

(U) The bacterium causing glanders was first isolated in 1882 by Loeffler and Schütz, according to Wilson and Miles,[2] by cultivating material from a horse dying from the acute disease. Actinobacillus mallei is a Gram-negative, non-motile rod measuring approximately 0.5 micron to 3.0 microns. When stained with aniline dyes it has a noticeable bipolar appearance. Sudan Black B may be used to demonstrate the presence of numerous intracellular fat droplets that may account in part for the irregular staining noted with use of other techniques.

(U) A. mallei is classified as an aerobe, and growth is markedly increased by aeration of liquid cultures by shaking or sparging. The characteristics of colonies on solid media vary considerably, depending on the strain and the medium on which it is growing. For example, on blood cysteine agar, colonies may have a pronounced grey color, but on the routinely used beef extract medium containing glycerol or sorbitol, they often have a yellowish color which varies in intensity with the strain and the age of the culture. Colonies of many strains demonstrate a tendency to wrinkle after aging for a few days. Several sugars and higher alcohols are used with resultant formation of acid without gas. Glycerol and sorbitol both enhance growth.

III. (C) EXPERIMENTAL STUDIES ON ACTINOBACILLUS MALLEI

A. (U) HISTORY OF A. MALLEI STRAINS

(U) Nineteen cultures of A. mallei were procured from various sources (Table I). The first six strains (C6 through C7) listed were obtained in September 1951 from Dr. Carl R. Brewer of the former Basic Sciences Division, Fort Detrick. Five of these strains were samples that had been lyophilized in normal animal serum in 1945 by personnel of the Miller research team of the former B Division.[1] Viable C7 strain cells could not be recovered from the growth on the single agar slant available. All six strains had been held at refrigerator temperature throughout the intervening years. Lyophilized cultures of strains 3709, 120-A, 7384, 3708, and 3873 were received from Dr. G. B. Reed of the Defence Kingston Laboratories of Canada in February 1952. No history accompanied these strains, but it was assumed that the one designated 3873 was identical with the C7 strain, because Miller[1] indicated that the latter was originally 3873 obtained from the U.S. Army Veterinary School. A subculture of 3873, designated 3873-18, was obtained from Dr. Reed in November 1952. This strain had been isolated as a single colony after passage in animals. Growth occurred in all cultures except 3709.

(C) Strains V, VII, VIII, and IX were received in 1952 from Dr. Francis B. Gordon of the former MV Division, Fort Detrick, who had received them from Captain T. Tulgo of the Turkish Army Veterinary Laboratory at Ankara, Turkey. These cultures had been isolated from human cases of glanders and were reported to be "suitable for the preparation of complement fixation antigens but not for agglutination tests." Some cultures gave rather striking and peculiar colonial forms on beef extract glycerol agar, including vivid yellow to amber color, mucoid consistency, and a marked tendency toward wrinkling.

(C) A period of approximately one year was spent in preliminary screening studies of these strains. Each one was cultured, passed a number of times through guinea pigs and hamsters, and the MLD or LD_{50} determined. Preliminary studies on resistance to drying, aerosol stability, and antibiotic therapy were also conducted.

(C) Because 3873-18 proved to be the only consistently virulent strain, it was selected for more detailed studies. The appearance of four-day-old colonies is shown in Figure 1. Unlike many of the other strains it did not produce rough or distinctly yellow colonies on beef extract glycerol agar.

(U) Strains AM-A, AM-B, and AM-C were obtained from Col M. B. Starnes of the Walter Reed Army Institute of Research, Washington, D. C. in 1957. No studies have been conducted with these three strains.

TABLE I. (U) DESIGNATION AND SOURCES OF ACTINOBACILLUS MALLEI STRAINS

DESIGNATION		PROCURED FROM	SOURCE	LOCATION	YEAR ISOLATED
Original No.	Detrick				
K	C6	Gen. Kelser, CI, Vet. Corps, US Army	- a/	-	-
3	C3	China-type culture collection via Gen. Kelser	-	Kweiyang	1942
5	C5	China-type culture collection via Gen. Kelser	Horse Lung	-	1942
2024 F22HP	2HP	US Army Vet. School	-	-	-
4	C4	China-type culture collection	Horse Lung	-	1942
3873	C7	US Army Vet. School	Fatal Human Case	-	1944
3709	3709	Dr. G.B. Reed, Canada	-	-	-
120-A	120-A	Dr. G.B. Reed	-	-	-
7384	7384	Dr. G.B. Reed	-	-	-
3708	3708	Dr. G.B. Reed	-	-	-
3873	3873	Dr. G.B. Reed	-	-	-
3873-18	3873-18	Dr. G.B. Reed	-	-	-
	V	Dr. F. Gordon, Fort Detrick	Human	Turkey	1949
	VII	Dr. F. Gordon	Human	Turkey	1951
	VIII	Dr. F. Gordon	Human	Turkey	1951
	IX	Dr. F. Gordon	Human	Turkey	1951
MM-A China 5 b/	AM-A	Col. Starnes, Walter Reed Army Institute of Research	Horse	Kweiyang	1942
MM-B 3873	AM-B	Col. Starnes	Human	Burma	1944
MM-C China 4 b/	AM-C	Col. Starnes	Horse	Kweiyang	1942

a. Unknown.
b. Probably the same as C5 and C4, respectively.

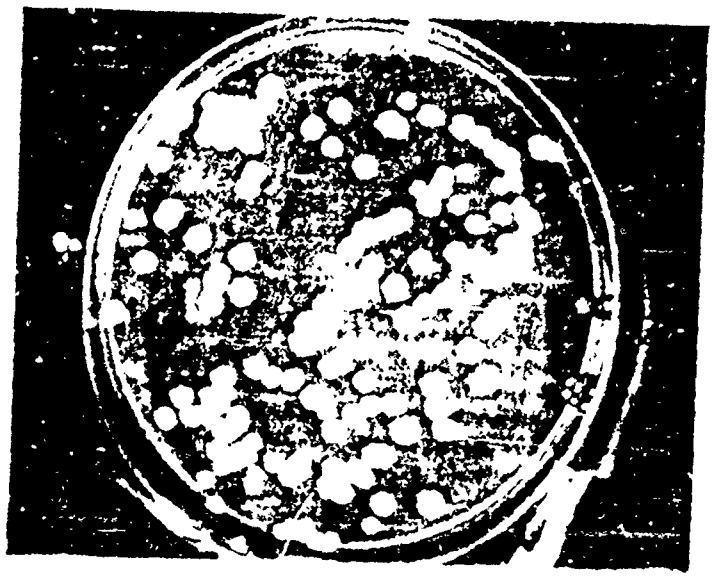

Figure 1. (U) Four-Day-Old Colonies of 3873-18.
(FD Neg C-3555)

B. (C) CULTURE PROCEDURES

(U) The liquid culture medium successfully employed for the growth of A. mallei by Miller[1] and other investigators had the following composition:

Beef Extract	0.3 per cent
Bacto Peptone	1.0 per cent
NaCl	0.5 per cent
Glycerol	4.0 per cent
pH adjusted to	6.8

This medium of beef extract glycerol (BEG) was adopted without extensive preliminary investigation on the basis of the high bacterial counts recorded. The addition of 1.5 per cent agar to the beef extract broth provided a solid medium for surface growth and colony counts. A temperature of 37°C was found optimum for incubation. Aeration by agitation on a reciprocating shaker for 48 hours gave bacterial counts of 1 to 2 x 10^{10}, compared with counts of approximately 1 x 10^{8} in stationary flask cultures. Agitation at constant temperature was accomplished in a water bath (Figures 2 and 3) mounted on a laboratory-type reciprocating shaker. Different sizes of Erlenmeyer flasks filled with liquid medium to 10 to 40 per cent of their capacities were used for 100 milliliters or less of culture. A special, low-form flask (Corning No. 4444) of 2000-milliliters capacity was used for volumes of 1000 to 1500 milliliters.

(C) Fifteen liters of culture were produced in five-gallon carboys in the following manner: Beef extract glycerol broth containing Dow-Corning Antifoam A was sterilized and cooled to 37°C. The medium was inoculated with a 24- or 48-hour broth culture in an amount equal to five to ten per cent of the volume of the medium. The inoculated medium was aerated during incubation with equipment pictured in Figure 4, with air supplied through an orifice at the rate of one-half liter per liter of culture per minute. Filtered, compressed air was introduced or filtered air was drawn into the growth vessel by applying negative pressure to the effluent air line. Maximum viable cell counts were obtained after 48 hours' incubation at 37°C. The resulting culture was very turbid and somewhat yellowish. The culture suspension was forced through sterile rubber tubing to a Sharples supercentrifuge and concentrated to a paste. Eighty to 120 grams of packed cells, containing 10^{11} to 10^{12} viable cells per gram, were usually obtained from 15 liters of culture.

(U) Concentrated cells of A. mallei were also obtained by inoculating BEG agar plates each with two-tenths milliliter of a liquid culture containing about 1 x 10^{10} viable cells per milliliter. After the plates had incubated for 48 hours at 37°C, the growth was scraped from the surface of the plate with a glass rake. Approximately eight milligrams of cells were obtained from each square centimeter of medium surface.

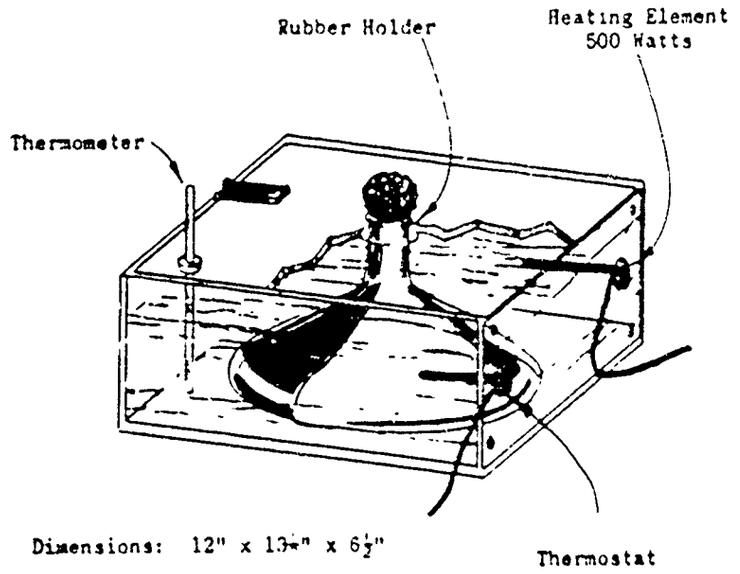

Figure 2. (U) Lucite Water-Bath Attachment for Reciprocating Shaker with 2000-Ml Culture Flask.

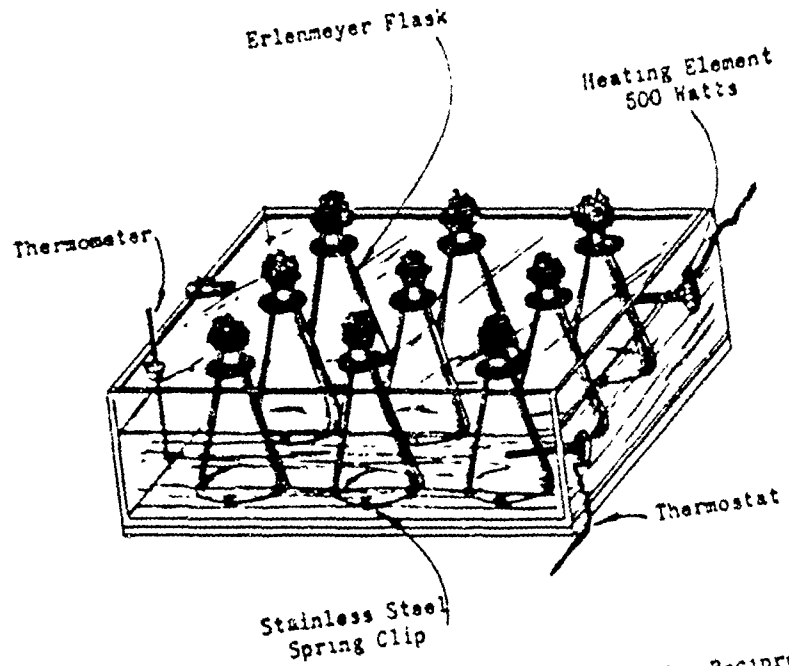

Figure 3. (U) Lucite Water-Bath Attachment for Reciprocating Shaker with 500-Ml Erlenmeyer Culture Flasks.

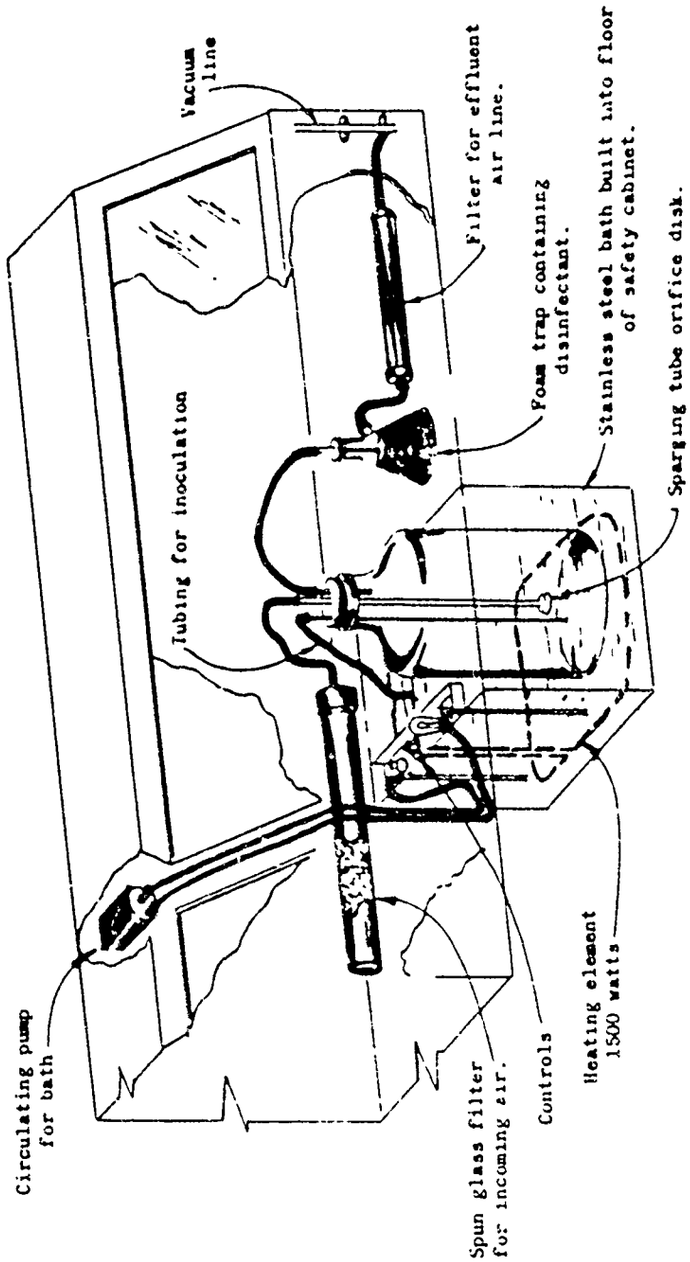

Figure 4. (U) Arrangement of the Five-Gallon Carboy Fermentation System in Class III Cabinet.

(U) In later studies, sorbitol was a satisfactory substitute for glycerol in the BEG medium. Optimum growth was obtained when two per cent (weight per volume) of crystalline d-sorbitol* was used in beef extract medium. Two other commercial sorbitol products gave unsatisfactory growth.

(U) The method for determining the viable cell count of A. mallei cultures was identical to that used by Miller et al,1/ which has been successfully applied to many other bacterial species at Fort Detrick. Ordinarily, tenfold dilutions of the suspension to be counted were made in distilled water blanks and those higher dilutions estimated to contain countable numbers of cells in one-tenth milliliter were used for seeding BEGA plates. One-tenth milliliter of the appropriate dilution was streaked over the surface of each of three agar plates with spreaders. Colonies of most strains could be counted conveniently after 48 hours' incubation at 37°C, although the more rapidly growing strains were sometimes ready for enumeration after 24 hours. All plates having less than 300 colonies were counted and the resulting figures were applied on a "weighted" basis** in calculating the average number of living cells per milliliter. Several commonly used diluting solutions were tested for their suitability in the quantitation procedure, but none offered any apparent advantage over distilled water.

C. (C) INFECTIVITY AND VIRULENCE STUDIES

(U) Shortly after receipt by SO Division, several colonies from each of the strains of A. mallei listed in Table II were picked and individually grown in BEG broth. Quantities of approximately 10^8 cells were injected intraperitoneally into male guinea pigs. Cultures of some strains killed the injected animals but others produced only chronic infections which were evidenced much later by healed lesions at the site of injection and/or microscopic evidence of epididymitis. None of the surviving animals developed a positive reaction to undiluted mallein when tested up to 40 days after infection.

(U) It soon became apparent that the guinea pig could not be relied upon as a test animal because response varied widely from test to test. The Strauss reaction was obtained infrequently and only in those animals in which a subacute or chronic disease was produced (Figures 5, 6 and 7). Canadian workers also reported unsatisfactory experience with the guinea pig.6/

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* Obtained from Atlas Powder Company, Wilmington, Delaware.
** The higher dilutions figured less prominently in deriving the average counts.

TABLE II. (U) EFFECT OF ANIMAL PASSAGE ON THE VIRULENCE OF VARIOUS ACTINOBACILLUS MALLEI STRAINS

STRAIN	INITIAL $IPLD_{50}$, 10^8 cells		FINAL $IPLD_{50}$[a], 10^8 cells	
	Guinea Pig	Hamster	Guinea Pig	Hamster
2MP	4.5		1.0	1.0
C6	1.0		1.0	1.0
C5	1.0		1.0	1.0
C4	1.0		1.0	1.0
C3	1.0		1.0	1.0
3708	5.2	1.5		9.0
7384	12.0			0.01
V	1.5	1.5		9.0
VII		1.5		5.3
VIII		1.5		
IX		1.5		1.0

a. After repeated passage in the two animals.

(U) The availability of hamsters shortly after this work was started provided a more uniformly susceptible and reliable test animal. The available strains were therefore passed in these animals several times by intraperitoneal injection followed by a virulence titration. For example, a smooth colony type of strain C-3 was carried through seven guinea pig passages (large numbers of organisms were necessary) and then through 13 hamster passages. The LD_{50} obtained in the subsequent virulence titrations in hamsters was still 10^8 viable bacteria. Similar results were noted for colonies of each of the other strains. As indicated in Table II, repeated animal passage without subculturing on media did not enhance virulence for either guinea pigs or hamsters. This was true of all colony types studied. In addition, aerosol challenge was usually not significantly effective in producing infection or death in hamsters and guinea pigs.

(U) Strain 3873-18 had a very high degree of virulence for the hamster. Previous investigators reported that the minimal intraperitoneal lethal dose of the lyophilized sample for hamsters was one to ten organisms; the respiratory dose was less than 20. These data were approximated and repeatedly obtained in many trials.

Figure 5. (U) Lateral View of Well-Developed Strauss Reaction. (FD Neg B-3965)

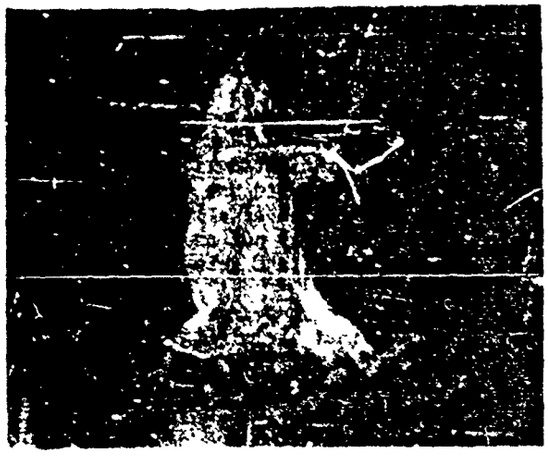

Figure 6. (U) Strauss Reaction with Necrosis of Scrotum and Fistula Formation. (FD Neg B-3968)

Figure 7. (U) Close-Up View of Testicles in Figure 6 Showing Extravasation of Liquefied Exudate as the Result of Incising the Scrotum. (FD Neg B-3967)

(C) A careful study of the colonial type indicated that 3873-18 had a marked degree of colonial stability, producing more than 90 per cent of the smooth, buff-colored colonies on BEGA medium (Figure 1). These smooth colony type organisms were uniformly virulent for the hamster and were selected for all subsequent studies with this strain. A total inoculum of 10^4 cells or higher usually killed the hamsters in 48 hours; lower numbers were lethal within ten days. Animals dying of the disease contained large numbers of organisms in the lungs, blood, and all visceral organs. Heart blood, liver, lungs, spleen, and testes were routinely examined and used for culturing to recover the organism. Animals dying within one or two days had acutely congested visceral organs and lungs. Those surviving for longer periods contained numerous caseous lesions in the various organs which tended to enlarge and coalesce if the animal survived for as long as six to ten days. The gross and microscopic pathology conformed in all respects to that described comprehensively in DRKL Report 41[8] and in Special Report 53.[7]

(U) No cases of chronic infection were produced with Strain 3873-18 in its initially virulent condition. Those rare animals that survived for more than ten days after injection with the highest dilutions (ten cells or less) showed no evidence of infection. Those receiving more than 100 viable cells by injection or aerosol almost invariably died within the first few days of holding.

(U) In early work with Strain 3873-18, aerosol trials were made primarily to determine its respiratory virulence in hamsters. The preparations tested included 48-hour undiluted BEG broth culture, 1 to 100 and 1 to 1000 dilutions of culture in BEG broth, and cell concentrates resuspended in distilled water. The cloud was generated by a Chicago-type atomizer connected to a constant feed device which provided a steady delivery of the slurry at a predetermined rate. Primary air for generating the cloud was supplied by a device for regulating the air pressure. The suspension was atomized into the 4800-liter test chamber. A fan with a 12-inch blade running at 230 revolutions per minute was activated during the time of aerosol generation and for a two-minute period thereafter to insure cloud homogeneity. Because of difficulty in handling hamsters it was necessary to place them in a hardware cloth cylinder which was then inserted into the aerosol chamber. Impinger samples of the aerosol were taken simultaneously with animal exposure to ascertain the concentration of organisms. Animals exposed to the same aerosol concentration were caged together. Calculation of the LD_{50} by aerosol inhalation cannot be considered precise because of the small numbers required for a lethal effect. However, a rather consistent death pattern was obtained with a given range of figures for the estimated inhaled dose (Table III). On the basis of these experimental data, the respiratory LD_{50} for hamsters is 27 viable cells, as calculated by the Reed-Meunch method.

TABLE III. (U) VIRULENCE OF ACTINOBACILLUS MALLEI, STRAIN 3873-18, FOR HAMSTERS CHALLENGED BY THE RESPIRATORY ROUTE

DOSAGE RANGE, viable cells	TRIALS, number	AVERAGE DOSE, viable cells	DEATH/TOTAL
1-10	4	4.5	4/12
10-100	6	60	27/32
100-1000	11	514	69/69

(U) Swiss-Webster mice (Detrick strain) were challenged by the intraperitoneal route with Strain 3873-18 of A. mallei with doses ranging from 20 to two million viable organisms. No deaths occurred in any of the injected animals during a 21-day holding period. At the termination of the experiment all animals were sacrificed and the lungs and visceral organs were cultured to detect any viable A. mallei organisms present. Because no evidence of infection was found, it is assumed that this species of rodents is quite resistant to this strain.

(U) Rhesus monkeys were injected intraperitoneally with a suspension of Strain 3873-18 containing 27,000, 14,000, and 1,400 cells, respectively. The only evidence of infection was the formation of an abscess at the site of injection in the animal receiving 27,000 organisms. No gross lesions were noted in the viscera or lungs when the animals were sacrificed 14 days later. In an experiment conducted to expose monkeys by the aerosol route to similar doses, only one monkey was exposed because of mechanical difficulties. It received an estimated 1,300 viable cells without showing evidence of infection. Because of the inability to establish infection, and because literature reports indicated that monkeys have a natural resistance to glanders, no further experiments were conducted with this animal.

(U) After approximately one year's work with Strain 3873-18, it underwent a marked drop in virulence that could not be explained. A number of animal passages were carried out and different colony selections were checked for virulence in an effort to derive a sub-strain having virulence approaching that of the parent strain. Selected isolates killed hamsters by intraperitoneal injection in rather low numbers, i.e., 20 to 100 organisms, but death patterns were erratic and virulence varied considerably from test to test. About the time this difficulty was encountered, Canadian workers experienced a similar drop in virulence for their cultures of this same strain.[1/] Additional cultures received from the

Canadians at a later date were also variable in their lethal effect for hamsters. Determinations of virulence made in 1958 on stored preparations of Strain 3873-18 indicated a further loss so that approximately 10^8 cells are required to kill hamsters by the intraperitoneal route.

(U) The prolonged and fruitless effort spent in trying to regenerate and to maintain high animal virulence in A. mallei by animal passage indicates the inherent difficulties. The value of this organism as a BW agent undoubtedly depends on acquisition of virulent strains or their development through genetic selection. Proven procedures for maintaining virulence are also needed. Even though such an undertaking has merit, the resources of Special Operations Division did not permit a research program of this scope. The loss in virulence reported here and noted by other workers was an important factor in the decision to discontinue the screening of A. mallei.[2,3]

(U) If a virulent strain of A. mallei were to be acquired or developed, the research reported in Sections III, D through III, H might be applied to the new strain to avoid duplication of that earlier work. For that possible future value, an account of the early work is included here.

D. (U) IN VITRO ANTIBIOTIC SENSITIVITY TESTS

(U) Several strains of A. mallei were tested for susceptibility to six commonly used antibiotics by two comparative procedures, (a) the tube test method, and (b) the impregnated disk method. The first procedure, the tube test method, consisted of incorporating different concentrations of an antibiotic in standard broth medium and inoculating the tubes with one-tenth milliliter of a freshly grown liquid culture of the organism. Control tubes of broth medium containing no antibiotic were also inoculated. Uninoculated tubes of broth served as negative controls. All tubes were observed after 24 and 48 hours' incubation at 37°C and compared turbidimetrically with the control tubes. Evidence of inhibition was most apparent in 24 hours' incubation; the minimum inhibitory concentration (MIC) increased twofold to fourfold at the 48-hour reading. Usually the 48-hour MIC level was the lethal concentration also, because living organisms could not be recovered from the tubes lacking turbidity. Table IV shows the MIC levels for the various strains studied. These findings were confirmed by duplicate tests. At low concentration chloramphenicol often inhibited multiplication for a short period, followed by a profuse overgrowth.

(U) Good correlation and confirmation of the tube tests were obtained with impregnated disks by using commercially prepared tabs* impregnated with various strengths of the respective antibiotics. The results are shown in Table IV. In this procedure a thin film of liquid culture was spread on BGA plates and a number of antibiotic disks were placed on the inoculated surface. The diameter of the area of inhibition was measured 24 and 48 hours after inoculation, and the MIC was determined by comparison with a chart supplied with the disks.

* Difco.

TABLE IV. (U) INHIBITION OF SELECTED STRAINS OF ACTINOBACILLUS MALLEI BY COMMONLY USED ANTIBIOTICS

ANTIBIOTIC	MINIMUM INHIBITORY CONCENTRATION OF ANTIBIOTIC, μg/ml										
	Strain										
	C3	C4	C5	C6	7384	3708	3873	125A	VII	VIII	IX
Tube-Test Method											
Penicillin	R[a]	R	R	R	R	R	R	R			
Oxytetracycline	1-5	1	1	1-5	5-10	1-5	1	1-5			
Chlortetracycline	1-5	1	1	1	1	1-5	1	1			
Chloramphenicol	1-5	1-5	1-5	1-5	5-10	R		1-5			
Streptomycin	5-10	1	10-20	1-5	1-5			1-5			
Dihydrostreptomycin	1-5	1-5	1-5	1-5	1-5			1-5			
Impregnated-Disk Method											
Penicillin						R	R				
Oxytetracycline	1.0	1.0			1.0-4.1	1.0-4.1	1.0-4.1		1.0	1.0	1.0
Chlortetracycline						1.0	0.6-4.9				
Chloramphenicol						5-10					
Dihydrostreptomycin						5-10					
Bacitracin						R	R				
Streptomycin							0.8-6.7				

a. Resistant.

(U) In the same test, chloramphenicol inhibited growth two to three hours before the organism grew around the disk. This might account for the discrepancy noted between the two methods for Strain 3708 (Table IV).

(U) Nigg and coworkers have indicated that the related organism, P. pseudomallei, may be differentiated from some strains of A. mallei on the basis of susceptibility to certain antibiotics; for instance, A. mallei is sensitive to streptomycin, P. pseudomallei is usually resistant to this antibiotic.[9]

E. (C) AEROSOL STABILITY

(C) Before the arrival of virulent Strain 3873-18, preliminary tests were carried out with other strains to learn something of the aerosol properties of A. mallei. For the most part the data were erratic. It was not readily apparent whether this inconsistency reflected variations in agent preparations or variations in mechanical and technical conditions in the test chamber, or a combination of both.

(C) Extensive aerosol studies were conducted with Strain 3873-18, the Strain selected for major emphasis. Liquid cultures of this organism were produced either in shake flasks or in a five-gallon carboy (described previously). The cell suspension (slurry) prepared by diluting a culture in broth was supplied to a Chicago-type atomizer through rubber tubing from a constant feed device. The slurry was atomized with compressed air at ten pounds per square inch in the 4800-liter test chamber maintained at 70°F and 50 per cent relative humidity. Aerosol recoveries from a large number of such trials varied considerably, although most were noted to fall about the mean of five to six per cent for the one-minute period after aerosolization. Subsequent changes in standard methods of aerosolizing and sampling, along with mechanical improvements in the tank, undoubtedly would provide more consistent data. Because of the marked variation in recoveries from test to test, it was also impossible to be certain of the effect of variations in temperature and humidity on the aerosols. The data suggest, however, that temperatures lower than 70°F and relative humidity values higher than 50 per cent would result in highest recoveries.

(U) The results of aerosol field trials with Strain 3873-18 conducted by Canadian workers of the Suffield Station in September 1953 was reported in August 1954.*

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* Trial Record 190, Suffield Experiment Station, Ralston, Alberta, Canada. SECRET 56-FD-TS-796.

F. (U) STORAGE STABILITY OF LIQUID SUSPENSIONS

(U) Data reported by Miller et al[1] indicated that optimal storage conditions were attained at 20 to 25°C by starting with dilute suspensions (10^7 cells per milliliter) in fresh broth and allowing slow growth during storage of several weeks. Some loss in virulence occurred during storage. When more concentrated suspensions (10^{10} cells per milliliter) were used for storage, a marked loss in viable cell count was noted within two weeks. The latter finding was verified by current work at BW Labs. Suspensions containing 10^9 viable cells per milliliter contained relatively few viable cells after two weeks' storage at -60, -20, 0, and 25°C. Instability of stored liquid cultures was also observed by the Canadians, who reported a drop in viable count and virulence of a suspension held for several weeks for wind tunnel trials.[7] In preliminary storage tests at 4°C the same group reported good stability for two to three weeks followed by a rapid fall the following week.

G. (U) DRYING STUDIES

(U) Contrary to results previously reported,[1] most of the available strains of A mallei were dried with reasonably good recoveries by two different methods.

(U) In the first method, thick suspensions of cells obtained from solid or liquid media were thoroughly mixed with an equal volume of five to 50 per cent egg white. The resulting preparation was spread in a thin layer on 18-inch by 18-inch pieces of safety glass and allowed to dry at room temperature under a stream of air from an electric fan. When the film appeared to be dry, it was removed with a hand scraper fitted with a single-edge razor blade insert. The resultant flaky material was collected on paper and stored in a glass container until it was ground.

(U) A 30 per cent viable recovery was usually obtained that resulted in material with counts of 10^{10} to 10^{11} cells per gram. This product was ground in a mortar and pestle during early work and later in the Tanner Spin Mill (Figures 8 and 9). Approximately 50 per cent of the viable count was lost during grinding by either method. Mortar grinding produced relatively large polyhedral, crystal-like particles which contained embedded bacterial cells. Particles from the Spin Mill treatment were round and more uniform in size. Stability studies with simulants indicate that egg white does not enhance stability but it does serve to separate bacterial cells and to permit grinding to a finely divided state.

(U) In the second method, suspensions of cells produced on solid medium or in a five-gallon carboy were thoroughly mixed with various diluents (equal volumes of cell concentrate and of suspending fluids). The resulting

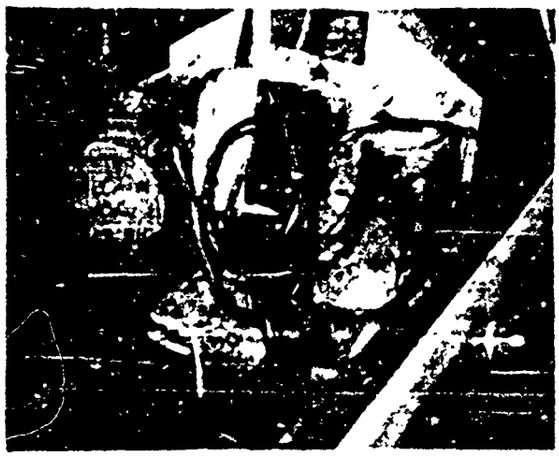

Figure 8. (U) Motors and Housing of Tanner Spin Mill Installed in Class III Safety Cabinet. (FD Neg C-3731)

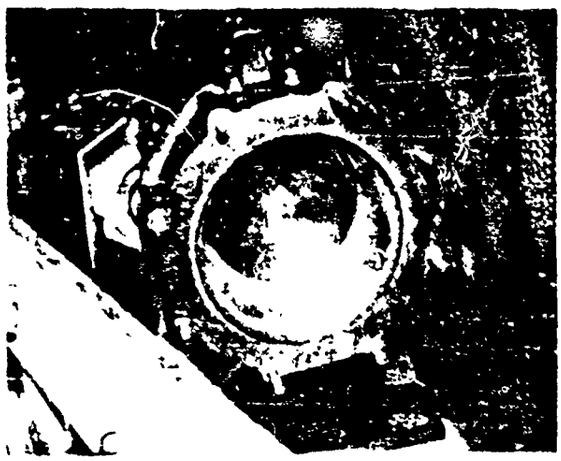

Figure 9. (U) Motor Housing of Tanner Spin Mill. (FD Neg C-3730)

liquid was poured into a Mylar-bottomed tray, frozen, and the frozen cell suspension was lyophilized in the freeze-dryer.* Lyophilization was carried out with or without infrared radiation from above and beneath the trays, Figures 10 and 11.

(U) Although the products from the above drying processes had somewhat greater storage stability than the liquid agent, their behavior as aerosols was disappointing. Virulence of the dry agent, determined by reconstitution and intraperitoneal injection, also was quite variable from lot to lot.

H. (U) HUMAN INFECTIONS

(U) A search of the literature for recent years revealed little detailed information on cases of human glanders with the exception of accidental laboratory infections at Fort Detrick. The six clinical cases were reported in the open literature[5] and detailed in Special Report 53.[1] Although these cases could be classified clinically as mild, the period of disability was lengthy. Available evidence suggests that the infecting dose probably was low. The extent of the pathological process also was probably limited in view of the reported clinical findings and the failure to isolate the infecting organism. In all six cases sulfadiazine proved to be an effective chemotherapeutic agent.

(U) In 1953, a moderately severe case resulted from an exposure of unknown source at Fort Detrick.[9] The 32-year-old typist infected did not work with the organism and ordinarily did not visit the laboratories. However, a laboratory accident several days before he became ill had allowed potentially contaminated material to escape from the Class III cabinet. Apparently the proximity of his office and the contaminated laboratory had allowed his exposure to the agent.

(U) The onset of symptoms was gradual and consisted of chest pains, ready fatigability, and night sweats. When first seen by the physician, he had a temperature of 101.2°F. A chest X-ray revealed an infiltrative process extending from the left hilum to the level of the first rib with a two-centimeter cavity in the second left anterior interspace. Only slight elevations in leukocyte count and erythrocyte sedimentation rate were noted throughout the illness. Other routine laboratory findings were essentially negative. Approximately eight days after his admission, the serum agglutinin titer for A. mallei was 1:640 and later rose to 1:2560. The complement-fixation test was negative. After his admission, numerous Gram-negative bacilli were isolated from the sputum and identified as A. mallei. The organism was (a) sensitive to therapeutically

* Dry-Freeze Corporation, Chicago, Illinois.

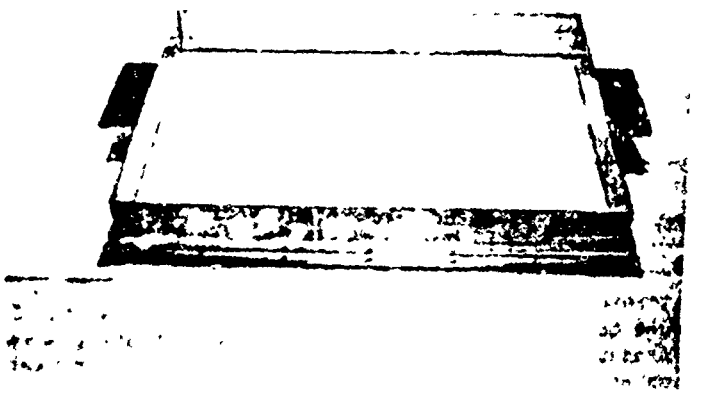

Figure 10. (U) Metal Lyophilizer Tray with Mylar Plastic Bottom. (FD Neg C-3732)

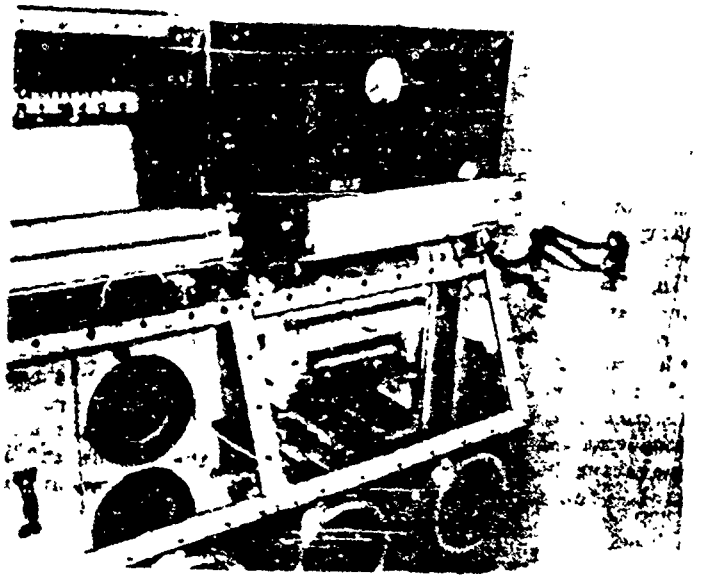

Figure 11. (U) Freeze-Dryer Apparatus and Controls Installed in Class III Cabinet. (FD Neg B-3523)

attainable levels of chlortetracycline and oxytetracycline, (b) moderately sensitive to streptomycin, and (c) resistant to penicillin, bacitracin and chloramphenicol. The mallein skin test was negative on repeated occasions. The patient was treated with chlortetracycline for 28 days during which time his temperature rose from 99 to 99.4°F almost daily. He was allowed up on the fourteenth day of hospitalization but continued to feel weak. After discharge, he was on sick leave for 12 days.

(C) The picture was complicated slightly by the fact that this patient reacted to coccidioidin (1 to 1000) by skin test, although previously his reaction had been negative. This finding led to the additional diagnosis of inapparent coccidioidomycosis.

(C) The glanders organism isolated from this patient possessed virulence for hamsters similar to that of Strain 3873-18, the one which presumably produced the infection. Later, the culture underwent a gradual decline in virulence and can now be considered without lethal effect for hamsters except in massive doses.

(U) This case report emphasizes the finding of Howe and Miller,[5] who also stated that the agglutination-titer determination is probably a more valuable clinical test for the diagnosis of glanders than the complement-fixation test.

IV. (U) PSEUDOMONAS PSEUDOMALLEI AND MELIOIDOSIS

A. (U) DISEASE HISTORY

(U) Melioidosis, a disease closely resembling glanders, was first recognized by Whitmore and Krishnaswami[10] in 1910 and reported in the literature in 1912. Between 1912 and 1947 slightly more than 300 cases were reported. It seems probable, however, that the disease has been more prevalent than these figures suggest, because a study of approximately 3,000 post mortems in Kuala Lampur between 1928 and 1930 revealed four and five-tenths cases per thousand.[11] The prohibition of post mortem examinations by various religious groups has probably prevented investigators from finding a greater incidence of the disease in various parts of the world. The varied manifestations of the disease and the difficulty in differentiating the causative agent from morphologically similar bacteria are also believed to have contributed to the small number of cases reported. The recognized disease is still uncommon, but a report of several cases in Indo-China was published in 1953;[12] in 1957 a case was diagnosed in an American serviceman stationed in Panama. Melioidosis has been reported from Australia, Burma, India, Federated Malay States, China, Thailand, Indo-China, Ceylon, Dutch East Indies, England, South Africa, Guam, North America, the West Indies, and Panama.[11-15]

B. (U) DISEASE DESCRIPTION

(U) Melioidosis in its acute form is usually fatal within a few days after a sudden onset. The symptoms and manifestations vary considerably, depending on the extent of the infectious process and the areas of the body affected. Clinically, it may simulate syphilis, smallpox, tuberculosis, malaria, cholera, glanders, and other diseases. Unlike glanders, melioidosis has been noted in epizootic form in laboratory rodents. The natural infection also has been seen in dogs, cats, horses, and sheep. Pathologically, the disease may be described as a pyemia closely resembling glanders in which the liver, spleen, lungs, skin, and other tissues and organs contain multiple small abscesses that frequently coalesce to form larger lesions. The chronic form is rare, but it often results in an illness with much suffering and marked incapacitation lasting for years before eventual recovery or death.[13,16]

C. (U) TRANSMISSION

(U) The reservoir of melioidosis is not known; although the natural infection has been seen occasionally in wild and laboratory rodents. Inland bodies of water have also been reported to contain the organism. Humans apparently derived the infection from the water.[17,18,20,21]

(U) Stanton and Fletcher[11] concluded that food and drink contaminated with rodent excreta were probably the sources of infection. However, Alain, St-Etienne and Reynes[19] found only one infected animal in 20,000 rats examined in Saigon, and Voucel[18] found none among 560 rats from the Tonkin area.

(U) Experimentally, Pseudomonas pseudomallei has been transmitted by the rat flea[9] and Aedes aegypti mosquito,[21] but it is not known whether any human infections have been contracted from these potential vectors.

(U) Man-to-man transmission has not been demonstrated, even though special precautionary measures were not taken in hospitals where patients with open lesions were attended, and in laboratories where cultures and infected animals have been kept.[11]

(U) Although the epidemiology of melioidosis is poorly understood, it seems reasonable to assume that the disease could be transmitted by a variety of means similar to those mentioned for glanders. The frequent involvement of the lungs in human cases and the known susceptibility of animals to air-borne organisms strongly suggest that the respiratory route was the avenue of infection in many instances. Patients demonstrating pulmonary localization with septicemia are considered to have a more grave prognosis than those with other forms of the disease.[12]

D. (U) PROPHYLAXIS AND THERAPY

(U) There is no vaccine of proven value for prophylaxis. However, autogenous vaccines are useful in the therapy of chronic cases.[22] Although certain commonly used antibiotics have proven effective in vitro, resistance in vivo has been observed to develop rapidly in certain instances.[23] At present, chloramphenicol is considered by investigators familiar with the disease as the drug of choice for treatment,[12,23] but the antibiotic spectrum of the particular strain would be a decisive factor in the choice of therapy. Most recent clinical trials using chloramphenicol in large, sustained doses indicate that the mortality rate may be substantially reduced if suitable antibiotic therapy is instituted early in the disease.[12]

(U) In many human cases of acute melioidosis the causative agent was not recognized before death.[24] Apparently those unfamiliar with the disease or the bacterium might regard isolates of P. pseudomallei as a contaminate, or confuse it with secondary pathogens similar in morphology and biochemical properties.

(U) Pathology of melioidosis resembles that of glanders except that the former is usually more extensive. A comprehensive compilation and description of the morbid anatomy of many human cases are found in the classic book of Stanton and Fletcher.[11]

E. (U) CAUSATIVE ORGANISM

(U) P. pseudomallei is a short, Gram-negative rod closely resembling the glanders bacillus; both have a bipolar appearance after staining with aniline dyes. Unlike the A. mallei, however, it exhibits serpentine motility, with its lophotricate flagella. It is also capable of fermenting certain carbohydrates to form small quantities of acid without gas. The incubation period for good growth of P. pseudomallei is about 24 hours, compared with that of 48 hours for A. mallei. Lein[25] has shown that the metabolic pathways of these two organisms also are divergent.

(U) Although no particular colonial type can be related to animal virulence,[11,28] rough or corrugated forms are isolated more frequently from human melioidosis.[11] In addition to the glanders bacillus, P. pseudomallei also is sometimes difficult to differentiate by laboratory tests from other species of Pseudomonas. Wetmore and Gochenour[27] suggested that certain biochemical reactions are useful in differentiating these species from one another.

V. (C) EXPERIMENTAL STUDIES ON PSEUDOMONAS PSEUDOMALLEI

A. (U) STRAINS OF P. PSEUDOMALLEI

(U) P. pseudomallei strains received at BW Labs from various sources are described in Table V. Virulence determinations were performed on the first eight strains listed, but only Strain 8016 has been used extensively in screening studies. The experimental data to follow were derived from the rough form of this strain except where otherwise indicated.

B. (U) CULTURE PROCEDURES

(U) The media and methods for growing A. mallei were suitable for P. pseudomallei, indicated by the work of Miller et al.[1] Both glycerol and sorbitol promoted growth in solid and liquid media. Maximum growth for A. mallei was obtained in about 18 to 24 hours in flasks or in five-gallon carboys by using the apparatus shown in Figures 2, 3 and 4. Counts of 1 to 3 x 10^{10} viable cells per milliliter for Strains W-294 and 8016 were routinely obtained under these conditions by using one to ten per cent inoculum from a fresh broth culture.

(U) In addition to the beef-extract base media previously described, the results of preliminary experiments indicated that casein acid digest (CAD) also can be used as a basic constituent of a liquid medium for the cultivation of P. pseudomallei. This medium contains the following ingredients:

Casein acid digest	0.85 mg of amino nitrogen per ml of final volume
Yeast (paste, Vico #75)	6.0 gm
KH_2PO_4	2.78 gm
K_2HPO_4	1.16 gm
Cerelose*	10.0 gm
Distilled water	to 1000 ml
pH	6.9

Counts as high as 6.0 x 10^{10} viable cells per milliliter were obtained in this medium after 24 hours' incubation at 37°C by using a one per cent inoculum.

(U) The subculturing of rough and smooth colonial forms of Strain 8016 in the CAD medium had no apparent effect on the virulence for hamsters infected by the intraperitoneal route (Table VI).

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* Cerelose added after sterilization.

TABLE V. (U) HISTORY OF PSEUDOMONAS PSEUDOMALLEI STRAINS HELD AT BW LABS

Original	Via	Designation Detrick	Via	NTL[a]	Originally procured from	Source	Location	Isolated Year	[illegible] cells
		1-295	[illegible] Kaiser	102	Calcutta School of Tropical Medicine	[illegible] strain	b/	b/	[illegible]
		1-294	[illegible] Kaiser	102	Calcutta School of Tropical Medicine	b/	b/	b/	[illegible]
		4014	Dr. [illegible] Defence [illegible] Kingston [illegible] Labs, Canada	[illegible]	British Type Culture Collection	b/	b/	b/	1-10
		1454	[illegible]	[illegible]	Army Med. Res. Unit, Saigon, Indo-China	b/	b/	1954	[illegible]
		1444	Col. [illegible]	[illegible]	Army Med. Res. Unit, Saigon, Indo-China	b/	b/	1954	[illegible]
		1459	Col. [illegible]	118	Army Med. Res. Unit, Saigon, Indo-China	b/	b/	1954	[illegible]
		1487	Col. [illegible]	110	Army Med. Res. Unit, Saigon, Indo-China	b/	b/	1954	[illegible]
		1488	Col. [illegible]	[illegible]	Army Med. Res. Unit, Saigon, Indo-China	b/	b/	[illegible]	[illegible]
Pf mallei		47-B	Col. W. B. Starnes, Walter Reed Army Inst. of Res., Wash. D. C.			Equine	Philippines	1947	
[illegible]		47-E	Col. W. B. Starnes			Equine	Malaya	b/	
304	[illegible] Kaiser	47-F	Col. W. B. Starnes	132		b/	b/	b/	
306	Jon. Kaiser	47-G	Col. W. B. Starnes	33		b/	China	b/	
C-3		47-H	Col. W. B. Starnes	104		b/	b/	b/	
1001		47-J	Col. W. B. Starnes			Human	Kuala Lumpur	1921	
4445		47-K	Col. W. B. Starnes			[illegible]	Singapore	1936	
4446		47-L	Col. W. B. Starnes			[illegible]	Singapore	1936	
[illegible]		47-M	Col. W. B. Starnes			Human	England	1942	
[illegible]		47-N	Col. W. B. Starnes			b/	b/	b/	
[illegible]		47-O	Col. W. B. Starnes			b/	b/	b/	
[illegible]		47-P	Col. W. B. Starnes			b/	b/	b/	
[illegible]		47-Q	Col. W. B. Starnes			Human	Burma	1945-46	
[illegible]	c/	47-R	Col. W. B. Starnes			Human	Colorado	1948	
300	d/	47-S	Col. W. B. Starnes			Human	Louisiana	1953	
		47-T	Col. W. B. Starnes			Human	Walter Reed [illegible]	1957	

a. [illegible] determinations by SO Division.
b. Unknown.
c. Isolated by [illegible], J.; [illegible], [illegible], and [illegible] "Characteristics of a strain of [illegible] Pseudomonas from Chronic Melioidosis," Am J Clin Path, 36 11 1341-1343 November [illegible].
d. Isolated by [illegible] and Fisher [illegible] "Chronic Melioidosis. Discussion Case Report and Special Studies," US Armed Forces Med J 9:6 1157-1160 January-June 1958

TABLE VI. (U) VIRULENCE AND VIABLE CELL COUNTS OF ROUGH AND SMOOTH COLONIAL FORMS OF P. PSEUDOMALLEI AFTER SUBCULTURE IN CASEIN ACID DIGEST BROTH

COLONY TYPE CULTURE	INITIAL VIABLE CELL COUNT, 10^{10}/ml	INITIAL LD_{50} cells	VIABLE CELL COUNT AFTER ONE TRANSFER, 10^{10}/ml	HAMSTER LD_{50} AFTER ONE TRANSFER, cells
Smooth	1.2	1.0	4.0	4.0
1-Rough	3.3	3.0	3.3	3.0
2-Rough	3.5	4.0	3.8	10
3-Rough	0.7	7.0	3.4	10

(U) Beef extract agar containing either glycerol or d-sorbitol was used as the routing solid medium to determine viable cell counts. Colonies can be counted on this agar after incubating for 48 hours at 37°C. Plates were incubated an additional four days at room temperature to distinguish colonial forms. Figures 12 and 13 show colony types obtained from Strain 8016. Figures 14 and 15 show the predominating colony types from two other strains grown on BEG agar medium.

(U) Distilled water and gelatin saline were as efficient as any of the several solutions tried as diluting fluids. However, in resuspending certain dried preparations it was necessary to add a surface-active agent, Tween 80, to the water. Distilled water and gelatin saline* are also satisfactory as aerosol impinger fluids.

C. (U) GENETIC STABILITY STUDIES

(U) The genetic stabilities of smooth and rough colony isolates from Strain 8016 were determined by subculturing them directly for six passages in beef extract sorbitol (BES) broth medium in shake cultures at 37°C. The final products were compared with the first passage culture with respect to viable count, colonial morphology, and virulence for hamsters by intraperitoneal injection.

(U) The smooth types did not give rise to rough colony forms, although the roughs reverted to smooth forms to a variable extent, depending on the particular isolate (Table VII). The smooth cultures also gave slightly higher counts in the shake-flask cultures, but the hamster virulence remained unchanged. These virulence data show no correlation between recognizable colony characteristics and virulence for the hamster.

* Two drops of olive oil served as an efficient antifoam agent.

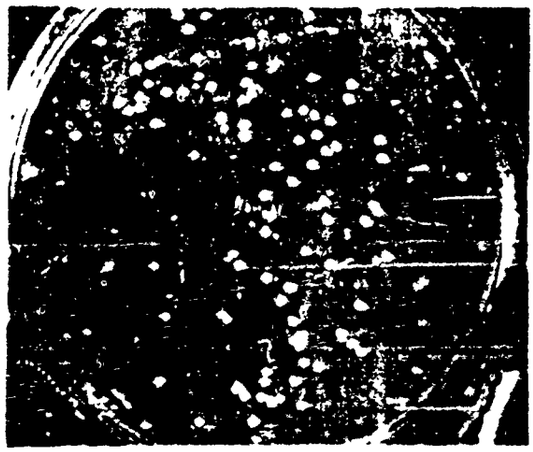

Figure 12. (U) Smooth Colonies of P. pseudomallei, Strain 8016, 96 Hours Old. (FD Neg C-3563)

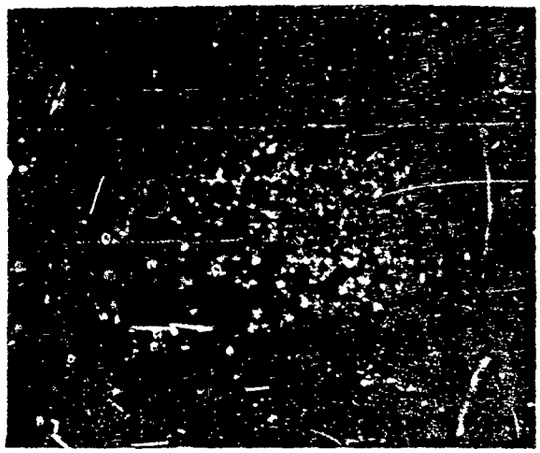

Figure 13. (U) Rough Colonies of P. pseudomallei, Strain 8016, 96 Hours Old. (FD Neg C-3565)

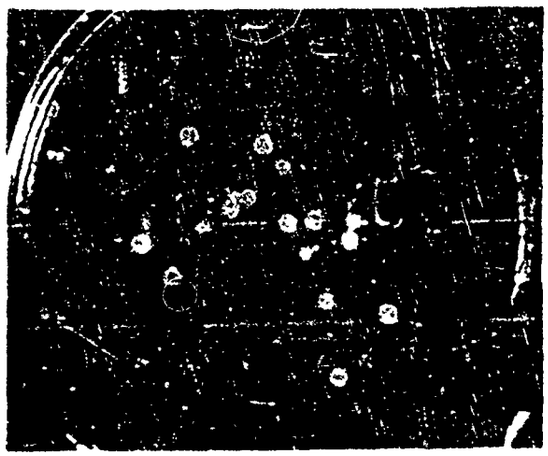

Figure 14. (U) Colonies of P. pseudomallei, Strain 1454, 96 Hours Old. (FD Neg C-3567)

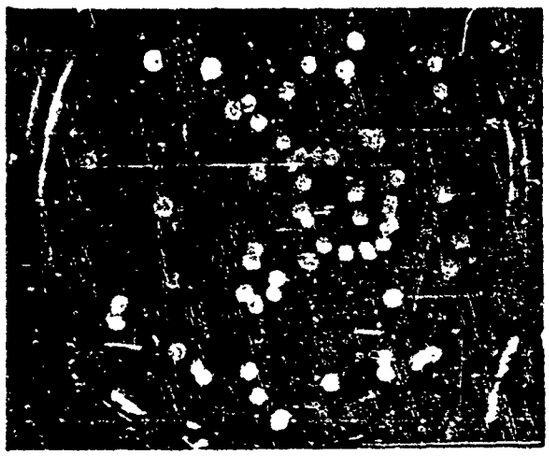

Figure 15. (U) Colonies of P. pseudomallei, Strain 1456, 96 Hours Old. (FD Neg C-3566)

TABLE VII. (U) VIABLE CELL COUNTS, VIRULENCE, AND COLONIAL MORPHOLOGY OF P. PSEUDOMALLEI AFTER SUBCULTURING IN BEEF EXTRACT SORBITOL BPOTH

ISOLATE	HAMSTER LD_{50}, cells		VIABLE CELLS, 10^{10}/ml		COLONIAL MORPHOLOGY, %			
	Initial	Fifth Passage	Initial	Fifth Passage	Initial S[a]	Initial R[b]	Fifth Passage S	Fifth Passage R
1-S[a]	2.0	< 8.8	4.75	5.77	100	0	100	0
2-S	3.0	< 8.6	5.04	5.20	100	0	100	0
1-R[b]	1.0	< 5.4	1.40	3.33	0	100	41	59
2-R	1.0	< 5.7	2.40	1.87	0	100	8.7	91.3

a. Smooth.
b. Rough.

(U) The effect of repeated subculture in BES broth on the virulence of cells was emphasized in another study with colonies selected at random. Ten colonies were picked from a sixth BES broth subculture and checked for hamster intraperitoneal virulence. A similiar number plated from a stock culture showed that all of the colonies were virulent for hamsters. These results indicate that this characteristic is relatively stable after an estimated 30 generations.

(U) Preliminary studies indicated that aeration of cultures by shaking or sparging is necessary for maximum growth. Therefore, the effect of varying degrees of oxygen tension on the growth and virulence of cells was investigated by using virulent products obtained in aerated shake flasks. Tube broth cultures were started with different quantities of inoculum and incubated for 48 hours at 37°C under static conditions. The viable cell count and virulence of the products are recorded in Table VIII. In most cases the lowest number of cells injected into hamsters was too high to obtain an LD_{50}. However, if the growth conditions used had decreased the virulance of this organism, one might reasonably expect the number of bacteria representing the LD_{50} value to have been much higher. The viable cell yield of the cultures incubated under static conditions was about two logs lower than that in the aerated cultures; again, the advantage of aeration is emphasized.

(U) Incubation temperatures ranging from 26 to 39°C supported cultures possessing high virulence for hamsters, although at the lower temperatures a longer period was required to obtain concentrations of 10^9 to 10^{10} cells per milliliter (Table IX). At 39°C the bacterial populations changed from rough to predominately smooth colony-forming types, which, as stated previously, do not differ in virulence for hamsters.

TABLE VIII. (U) EFFECT OF OXYGEN TENSION ON THE GROWTH AND VIRULENCE OF P. PSEUDOMALLEI IN BEEF EXTRACT SORBITOL BROTH

GROWTH CONDITION	VIABLE CELL COUNT OF INOCULUM, cells/ml	VIABLE CELL COUNT AFTER INCUBATION, cells/ml	HAMSTER LD_{50}, cells
Aerated, shake flasks	3.0×10^8	2.3×10^{10}	< 23
	3.0×10^7	1.6×10^{10}	< 16
	3.0×10^6	9.4×10^9	< 9
	3.0×10^5	5.2×10^9	< 52
Static (without oil)	3.0×10^8	1.1×10^8	< 110
	3.0×10^7	1.0×10^8	< 100
	3.0×10^6	4.0×10^8	< 40
	3.0×10^5	1.4×10^8	< 140
Static (with layer of oil)	3.0×10^8	8.1×10^7	< 81
	3.0×10^7	8.1×10^8	< 110
	3.0×10^6	5.0×10^7	< 5000
	3.0×10^5	5.0×10^7	< 5000

(U) Table X contains the data from experiments in which BSG broth with and without a layer of paraffin oil and Brewers' thioglycollate medium were used to culture the organism at 37°C. Virulence of cells from each culture was determined in the usual manner after three weeks of incubation. No significant differences in virulence were detected as the result of the prolonged incubation.

TABLE IX. (U) INFLUENCE OF INCUBATION TEMPERATURE ON GROWTH AND VIRULENCE OF AERATED BROTH CULTURES OF P. PSEUDOMALLEI

INCUBATION		VIABLE CELL COUNT		HAMSTER
Temperature, °C	Hours	After Inoculation, cells/ml	After Incubation, cells/ml	LD_{50}, cells
26	48	5.2×10^6	1.3×10^{10}	28.0
		5.2×10^5	2.2×10^9	5
		5.2×10^4	1.1×10^9	22
		5.2×10^3	1.1×10^9	22
30	48	2.1×10^6	7.3×10^9	1.0
		2.1×10^5	1.0×10^{10}	20
		2.1×10^4	9.0×10^9	120
		2.1×10^3	1.2×10^9	27
39	24	1.4×10^5	2.3×10^{10}	5.0
		1.4×10^4	2.2×10^{10}	5.0
		1.4×10^3	2.0×10^{10}	4.0
		1.4×10^2	2.5×10^{10}	7.0

TABLE X. (U) VIRULENCE OF BROTH CULTURES OF P. PSEUDOMALLEI AFTER PROLONGED INCUBATION UNDER STATIC CONDITIONS AT 37°C

GROWTH MEDIUM	VIABLE CELL COUNT OF INOCULUM, cells/ml	VIABLE CELL COUNT AFTER 3 WEEKS, cells/ml	HAMSTER LD_{50}, cells
Thioglycollate broth	1.7×10^5	1.6×10^6	28
	1.7×10^4	3.8×10^6	1
	1.7×10^3	7.4×10^6	16
	1.7×10^2	3.6×10^6	11
Beef glycerol broth (without oil)	1.4×10^4	3.9×10^5	--
	1.4×10^3	6.0×10^6	19
	1.4×10^2	6.8×10^6	21
	14	2.0×10^6	3
Beef glycerol broth (with layer of oil)	1.4×10^4	4.9×10^7	15
	1.4×10^3	2.7×10^6	6
	1.4×10^2	2.8×10^6	1
	14	7.8×10^6	2

D. (U) VIRULENCE STUDIES

1. (U) Hamster

(U) A large number of virulence titrations using P. pseudomallei, Strain 8016 indicate that the hamster intraperitoneal LD_{50} for this strain is between one and ten cells and the respiratory LD_{50} for this animal is between four and twenty cells. Hamsters given high doses (15×10^6 organisms) by the oral route died from melioidosis; others receiving 15×10^3 and 15×10^1 cells by this route showed no evidence of infection when sacrificed 15 days later. Thus, oral infectivity for hamsters does not appear to be of a high order; this finding was reported by the NBL group.[26]

(U) The gross and microscopic pathology studies carried out on hamsters infected with P. pseudomallei agreed closely with the findings of several other groups.[1,28] As would be expected, the lungs of aerosol-exposed animals usually contained a greater number and more extensive lesions than those of animals injected or fed the virulent bacilli. However, these organs were almost always involved to some extent in the infection regardless of the route of entry. In addition to the characteristic lesions pictured in Figures 16 and 17, many of the organs showed either hemorrhage or marked congestion with edema. Animals surviving the first two or three days post-infection often were observed to have a discharge from the eyes and an opacity of the corneas that resulted in blindness.

(U) Giemsa or other appropriate stains for bacteria revealed numerous intracellular and extracellular bacteria in the lesions which were somewhat longer than the forms seen in broth cultures. The marked tendency for the necrotizing lesions to enlarge and coalesce and the miliary aspects of the lesions in the lungs, spleen, and liver further emphasize the fact that the hamster has very little resistance to P. pseudomallei infection.

2. (U) Guinea Pig

(U) The majority of the reports in the literature regarding the pathogenicity of P. pseudomallei indicate that the guinea pig is very susceptible to infection with this organism. Publications originating at Fort Detrick and the Naval Biological Laboratories in Berkeley, California indicate that the LD_{50} by the intraperitoneal route is approximately 500 viable organisms. Other sources, however, report that the guinea pig varies in its response to infection with P. pseudomallei, i.e., certain animals do not die when injected with concentrations of bacteria exceeding the usual LD_{50} figure. The results of two typical experiments with the Hartley line of male guinea pigs* (350 grams) suggest that Strain 8016 has an LD_{50} of about 50 organisms by the intraperitoneal route of challenge, and an LD_{50} of about one by the subcutaneous route (Table XI)

* Fort Detrick stock.

Figure 16. (U) Portion of a Lesion of Melioidosis Adjacent to a Vein in the Lung of a Hamster. (FD Neg C-1555)

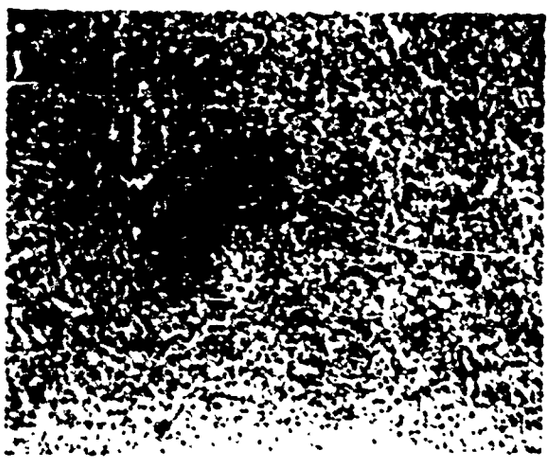

Figure 17. (U) Small, Coalescing Lesions of Melioidosis in Liver of Hamster. (FD Neg C-1556)

TABLE XI. (U) VIRULENCE OF P. PSEUDOMALLEI, STRAIN 8016, FOR GUINEA PIGS AND HAMSTERS

ANIMAL	ROUTE OF INOCULATION	LD_{50}, cells	
		Experiment 1	2
Guinea Pig	Intraperitoneal	52	54
Guinea Pig	Subcutaneous	1	1
Hamster	Intraperitoneal	4	1

(U) In these experiments, virulence was determined by using hamsters as a control. All animals were held for 14 days. Those animals that expired four to five days after inoculation were autopsied, and death from melioidosis was confirmed by culturing the heart, lungs, liver, and spleen. Colonies typical of P. pseudomallei were recovered from all dead animals. The Strauss reaction was noted in all male guinea pigs dying after the third day of infection.

(U) In further studies, more variable results were obtained by using mixed groups of animals of different sizes and ages; an occasional animal resisted the lethal effect of as many as 10,000 cells for more than the two-week holding period.

(U) Microscopic examination of various tissues and organs of guinea pigs which died from the infection revealed a lesion similar to that noted in hamsters. However, the number of lesions was less and fewer extracellular bacilli were present in those lesions. In many cases, the extent of the infectious process did not seem to justify the death of the animal. It seems logical to assume that a toxemia resulting from proliferation of the organisms has much to do with the fatal termination. Such a phenomenon probably obtains in human cases also, where the extreme emaciation described is far out of proportion to the infectious process.[12,19]

3. (U) Monkeys

(U) Rhesus and cynomologus monkeys injected intraperitoneally with 30 to 50,000 viable cells of P. pseudomallei showed no evidence of progressive infection. However, an abscess was produced at the site of injection of the monkeys receiving the highest dose (50,000 organisms). This abscess healed within the holding period of two weeks. Gross examination of the lungs and viscera failed to reveal any lesions attributable to P. pseudomallei. Because the first six virulent strains (Table V) gave similar results, it was concluded that both species of monkey were relatively resistant to infection with P. pseudomallei by the intraperitoneal route.

(U) Three monkeys were successfully infected with P. pseudomallei, Strain 8016, when challenged by the oral route with large numbers of organisms. One, fed a biscuit containing approximately 10^{10} viable cells, died 14 days later of melioidosis. The other two monkeys, injected with approximately 10^{9} viable organisms directly into the stomach by means of a catheter, were infected with P. pseudomallei as evidenced by an increase in agglutination titers, an increase in the number of circulating white blood cells, and by the isolation of P. pseudomallei at the time of sacrifice.

E. (C) AEROSOL STABILITY STUDIES

1. (C) General Procedure

(C) Many aerosol tests have been carried out with P. pseudomallei, Strain 8016. In general, the results indicate that this organism is at least as stable in aerosol form as certain other vegetative pathogenic bacteria, i.e., Pasteurella tularensis, Pasteurella pestis, and certain species of Brucella.

(C) A series of aerosol runs was made with beef extract sorbitol broth cultures of P. pseudomallei grown in 1500-ml quantities in 2000-ml Erlenmeyer flasks on a water-bath shaker at 37°C. Fifteen hundred milliliters of broth were given a ten per cent inoculum from a culture containing 5×10^{9} cells per milliliter. After 24 hours' incubation, a sample was withdrawn for aerosol recovery determinations and viable cell counts. A ten per cent inoculum from this culture was used to seed another 1500 milliliters of beef extract sorbitol broth. Fresh cultures were provided daily for three days for aerosol evaluation. The material was aerosolized into a 4800-liter test tank by means of a specially constructed spray-gun device, FK8 (Figure 19). From simulants, the FK8 has produced small-particle aerosols ranging from two to five microns in diameter with a uniformly high recovery rate. No significant difference was found between production batches of the liquid agent nor was any effect noted with change in relative humidity. A summary of the data from these trials is shown in Table XII.

(U) Lots 1 through 5 were produced serially in 1500-ml shake flasks by using inoculum from the preceding lot. In Lots 6 through 8, however, several seed flasks were prepared as follows: A 300-ml Erlenmeyer flask containing 25 ml of beef extract sorbitol broth was inoculated with a loopful of growth from an agar slant culture of P. pseudomallei. The flask was incubated on a water-bath shaker for 24 hours at 37°C. Four to eight 300-ml Erlenmeyer flasks containing 24 ml of broth each were inoculated with one milliliter of inoculum from the above broth culture. These flasks were incubated on a shaker for 24 hours at 37°C and stored at 4°C until used. A 2000-ml flask containing 1500 ml of beef sorbitol broth was inoculated with 24 ml of culture from a seed flask. After 24 hours' incubation at 37°C, samples of the culture were assayed for aerosol properties. The aerosol decay rate for these three lots of material averaged about the same as those for the five previous lots.

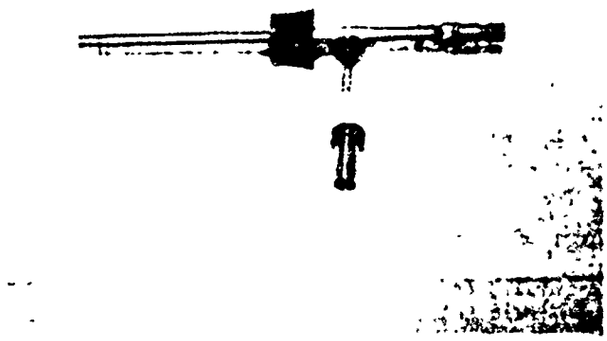

Figure 18. (U) Disassembled FK8 Aerosol Dissemination Device Showing Slurry Reservoir. (FD Neg C-3733)

TABLE XII. (C) AEROSOL STABILITY OF P. PSEUDOMALLEI, STRAIN 8016, GROWN IN BEEF EXTRACT SORBITOL BROTH IN 1500-ML QUANTITIES

LOT	VIABLE CELLS/ml	PER CENT AEROSOL RECOVERY, min 0•2	0•16	0•30	DECAY RATE, %/min
1[a]	5.71×10^9	41.9	19.3	13.0	4.18
2[b]	5.71×10^9	44.0	20.6	11.6	4.76
3[c]	6.10×10^9	46.5	20.6	13.4	4.44
4[c]	1.00×10^{10}	27.6	11.4	7.5	4.65
5[c]	6.00×10^9	28.5	13.7	10.5	3.74
6[c]	7.10×10^9	30.7	6.2	4.6	6.80
7[c]	1.51×10^{10}	34.1	18.7	10.8	4.10
8[c]	1.20×10^{10}	23.8	16.4	13.0	3.40

a. Average of nine independent trials at 50 per cent relative humidity.
b. Average of nine independent trials at 80 per cent relative humidity.
c. Average of six independent trials at 50 per cent relative humidity.

2. (C) Effect of Storage on Aerosol Stability.

(C) The results from one series of experiments with three lots of P. pseudomallei, strain 8016, grown and stored in beef extract sorbitol broth, indicate a progressive drop of aerosol stability during storage. The experiment was performed as follows: A loopful of growth from a beef glycerol agar slant culture started from a single rough colony was used to seed 25 ml of beef extract sorbitol broth in a 300-ml Erlenmeyer flask. The flask was incubated for 24 hours on a water-bath shaker. One-milliliter aliquots of this culture were used to inoculate four 300-ml Erlenmeyer flasks containing 24 ml of beef sorbitol broth. These four seed flasks were incubated with aeration for 24 hours at 35 to 47°C and then held at cabinet temperature. A 2,000-ml flat-bottomed flask containing 1500 ml of beef sorbitol broth was inoculated with 25 ml of culture from a seed flask and incubated at 35°C for 24 hours on a water-bath shaker. Three lots (designated A, B, and C) of P. pseudomallei were produced in this manner on alternate days. Samples from each of the three lots were assayed in aerosol form at 0, 2, 4 and 8 weeks. The clouds were disseminated into a 4800-liter test tank with FX8. The broth cultures were stored in sterile 500-ml heavy-wall reagent bottles at 5°C.

(U) Table XIII gives the initial viable cell counts of the three lots of P. pseudomallei as determined by four different groups in SO and Technical Evaluation Divisions. The three lots had about the same viable cell count.

TABLE XIII. (U) VIABLE CELL COUNTS OF THREE LOTS OF P. PSEUDOMALLEI GROWN IN 1500-ML QUANTITIES IN BEEF EXTRACT SORBITOL BROTH[a]

LOT	INOCULUM, 10^8 viable cells/ml	VIABLE CELL COUNTS, 10^9/ml				AVERAGE VIABLE COUNT, 10^9
		SO Division		TE Division		
		M Branch	Eval Branch	A&E Sect	MR&AE Br	
A	2.5	10.0	12.3	10	7.5	9.9
B	1.4	8.5	9.17	7.0	7.2	7.9
C	3.0	10.3	8.7	7.4	8 8	8.8

a. Cultures incubated on a water-bath shaker for 24 hours at 35°C.

(U) Data concerning the physical properties of the three lots of material are compiled in Table XIV. All three had about the same pH and specific gravity.

TABLE XIV. (U) PHYSICAL PROPERTIES OF THREE LOTS OF P. PSEUDOMALLEI GROWN IN BEEF EXTRACT SORBITOL BROTH AND TESTED AT 30°C

LOT	VISCOSITY		SPECIFIC GRAVITY	SURFACE TENSION, dynes/cm	pH
	CSTKS[a]	CPS[b]			
A	1.07	1.09	1.017	41.7	7.6
B	1.14	1.16	1.014	48.0	7.7
C	1.006	1.021	1.015	49.1	7.7

a. Centistokes.
b. Centipoises.

(U) The aerosol recovery values of the fresh slurry of Lots A, B and C of P. pseudomallei are shown in Table XV.

TABLE XV. (C) AEROSOL RECOVERY VALUES[a] OF THREE LOTS OF P. PSEUDOMALLEI GROWN IN BEEF EXTRACT SORBITOL BROTH

LOT	DAY	VIABLE CELL COUNT per ml	PER CENT AEROSOL RECOVERY, min 0·4	0·8	0·32
	1	1.23×10^{10}	28.6	4.3	3.4
	1	1.23×10^{10}	32.7	5.4	2.9
A	2	1.28×10^{10}	--	2.0	2.1
	2	1.28×10^{10}	11.5	2.1	1.4
	3	9.17×10^{9}	9.1	3.9	2.3
	3	9.17×10^{9}	49.8	9.3	3.6
B	4	6.93×10^{9}	21.8	4.7	4.4
	4	6.93×10^{9}	24.6	6.9	3.6
	5	8.7×10^{9}	22.2	7.2	7.2
	5	8.7×10^{9}	26.4	3.8	2.7
C	6	7.18×10^{9}	26.5	6.2	2.8
	6	7.18×10^{9}	28.4	4.5	2.8
Average			25.6	5.0	3.3
Decay Rate			7.6 per cent/min		

a. FK8 atomizer used. Determinations made by GO Division.

(C) The effect of storage at 5°C on the aerosol stabilities of the three lots of P. pseudomallei is shown in Table XVI. The cloud decay rates were similar for all three lots of material after two and four weeks' storage. However, the average source strength (per cent recovery) at 0·4 minutes for the three stored lots, (excluding Lot B at the two-week test period), was roughly one-half that of the fresh cultures. This effect of holding broth cultures at 5°C on the aerosol recovery of the agent needs further investigation.

3. (C) Comparison of Aerosols from Two Disseminators

(C) The same three lots of P. pseudomallei were used in a cooperative study (with Technical Evaluation Division) of the FK8 and the C generator. The source strength of the aerosol varies with the disseminating device used. The aerosols produced by the C generator were about five times lower in source strength than those from the FK8 (Table XVII). Also, the

TABLE XVI. (C) AEROSOL STABILITY OF THREE LOTS OF P. PSEUDOMALLEI GROWN IN BEEF EXTRACT SORBITOL BROTH AND STORED FOR SEVERAL WEEKS AT 5°C.a/

RUN	LOT A AEROSOL RECOVERY, % Min. 0/2	0/16	0/30	RUN	LOT B AEROSOL RECOVERY % Min. 0/2	0/16	0/30	RUN	LOT C AEROSOL RECOVERY, % Min. 0/2	0/16	0/30
					TWO WEEKS						
2558	9.0	1.7	1.0	2564	21.0	1.3	0.8	2570	6.7	0.4	0.3
2559	8.2	1.3	0.7	2565	21.2	1.1	1.0	2571	2.5	0.3	0.2
2560	10.8	1.7	1.0	2566	17.9	1.8	0.5	2572	9.2	0.5	0.4
2561	8.4	1.1	0.6	2567	32.7	1.0	0.3	2573	9.2	1.0	0.8
2562	11.6	1.2	0.9	2568	25.5	2.0	1.4	2574	9.4	0.6	0.5
2563	12.7	1.2	0.9	2569	34.7	2.7	1.2	2575	15.4	1.4	0.7
Average	10.1	1.4	0.9		25.5	1.7	0.8		8.8	0.7	0.5
Decay Rate	8.6%/min				11.8%/min				10.2%/min		
Viable Cell Count/ml	6.35 x 10^9				6.57 x 10^9				8.1 x 10^9		
					FOUR WEEKS						
2619	9.8	1.0	0.3	2625	9.4	0.5	0.4	2631	7.5	0.4	0.3
2620	15.5	1.5	0.4	2626	2.7	0.9	0.4	2632	13.4	0.5	0.3
2621	16.3	1.0	0.3	2627	13.2	0.8	0.4	2633	5.3	1.8	0.8
2622	14.4	0.7	0.3	2628	22.4	1.4	0.6	2634	8.3	2.0	1.1
2623	17.8	1.3	0.3	2629	7.8	2.7	1.5	2635	6.0	2.1	1.3
2624	11.3	1.1	0.5	2630	10.3	2.0	1.2	2636	9.7	1.5	1.1
Average	14.2	1.1	0.4		11.0	1.4	0.8		8.4	1.4	0.8
Decay Rate	12.7%/min				9.4%/min				8.4%/min		
Viable Cell Count/ml	6.05 x 10^9				6.85 x 10^9				8.37 x 10^9		
					EIGHT WEEKS						
2685	3.3	0.4	0.2	2691	1.7	0.5	0.5	2697	1.7	0.7	0.3
2686	4.8	0.2	0.3	2692	4.0	0.5	0.4	2698	2.7	0.5	0.4
2687	4.2	0.3	0.2	2693	4.6	1.7	0.8	2699	3.7	1.0	0.8
2688	6.0	0.3	0.3	2694	5.8	2.3	1.4	2700	7.1	1.3	1.0
2689	3.6	0.7	0.3	2695	7.0	2.3	1.0	2701	3.1	1.2	0.9
2690	3.8	0.6	0.2	2696	3.7	3.3	2.1	2702	7.4	1.2	1.1
Average	4.3	0.4	0.3		5.4	1.8	1.2		4.3	1.0	0.8
Decay Rate	9.5%/min				6.8%/min				6.0%/min		
Viable Cell Count/ml	6.2 x 10^9				5.4 x 10^9				7.3 x 10^9		

a. Determinations made by SD Division.

source strengths of Lots A and B disseminated by the C generator were significantly higher at the P=.05 level. It was concluded that the lower source strength of Lot C disseminated by the C generator was a result of the day rather than of the lot, because data derived from dissemination of simulant on the same day also were significantly lower than that for the other two days of testing. Because results with Serratia marcescens gave the expected high recovery values when the C generator was used, it was concluded also that the generator exerted a deleterious effect on the pathogen; this effect was not evident with the FK8 device.

TABLE XVII. (C) MEAN SOURCE STRENGTHS AND DECAY RATES OF P. PSEUDOMALLEI GROWN IN BEEF EXTRACT SORBITOL BROTH AND DISSEMINATED BY TWO METHODS

LOT	AEROSOL RECOVERY, PER CENT 0 ∕ 4 min.		DECAY RATE Per cent/min.	
	FK8	C Generator[a]	FK8	C Generator
A	24.3	8.0	3.46	5.07
B	35.4	7.1	2.91	3.10
C	25.9	6.8	1.48	3.53

a. Determinations by MR and AE Branch, Technical Evaluation Division.

(C) The results obtained by using the C generator indicate that the source strengths of the three lots of P. pseudomallei after 24 hours' storage at 4°C were significantly lower than those of the freshly produced agent (Table XVIII). This confirms the previous finding that storage of broth suspension of this organism at 4°C adversely affects aerosol stability. Additional data on these agent device trials are contained in the Technical Evaluation Division Report of Test 57-A-905.[29]

(U) A comparison of the aerosol properties and respiratory infectivity of rough and smooth strains of P. pseudomallei grown in beef extract sorbitol medium and in casein acid digest medium was determined by Technical Evaluation Division. Both smooth and rough strains grown in the beef sorbitol medium exhibited significantly higher source strengths than when cultivated in the CAD medium. The smooth strain cultured in beef sorbitol showed higher source strengths and lower decay rates than the rough strain grown in the same medium.

TABLE XVIII. (C) MEAN SOURCE STRENGTHS AND DECAY RATES OF P. PSEUDOMALLEI GROWN IN BEEF SORBITOL BROTH AND STORED FOR 24 HOURS AT 4°C

TIME OF TRIALS	RECOVERY, PER CENT 0 / 4 min. Lots			DECAY RATE %/min. Lots		
	A	B	C	A	B	C
Day						
1	5.00	4.56	2.03	5.66	2.62	2.53
2	2.40	1.85	1.08	4.46	3.60	4.50

(C) The respiratory LD_{50} for guinea pigs and hamsters of the various strains cultured in the two media could not be estimated because a high rate of kill was experienced with both species of animals. However, the rough strain grown in CAD produced 100 per cent mortality with fewer organisms than the smooth or rough strain cultured in beef sorbitol. The experimental procedure and test details are given in Technical Evaluation Division Report of Test 58-A-956.[30]

F. (C) DRYING STUDIES

(C) In initial studies conducted with P. pseudomallei in SO Division, virulence decreased during the drying process. Several suspending fluids were studied for their ability to enhance stability and to retain virulence of the agent through the lyophilization process, but none of those tried were superior to distilled water. The cultures for these early studies were produced in 15-liter amounts of beef extract glycerol or sorbitol broth in the five-gallon carboy sparger. Cells from the cultures were centrifuged in a Sharples Supercentrifuge. The "mud" recovered usually had a count of 10^{11} viable cells per gram, although some lots yielded as much as 10^{12} viable cells per gram. The cell concentrate was then resuspended in an equal volume of a suspending fluid, placed in drying trays, frozen at -60°C, and lyophilized in the freeze-dryer. Dried products obtained from this procedure frequently had a count of 10^{11} viable cells per gram. Grinding of the dry product in a Tanner Spin Mill reduced the viable count by 30 to 80 per cent.

1. (U) Mechanism of Virulence Loss

(U) Recent drying studies were directed toward elucidating the mechanism of virulence loss occurring in the lyophilization process. The results obtained from four replicate experiments indicate that the reduction was caused by a temporary physiological change and not by a genetic alteration.

(U) The experiments were designed to test two hypotheses. (a) If the reduction in virulence during drying was caused by a genetic change, the dried product would contain a large proportion of avirulent cells. Hence, broth cultures started with inocula which had undergone the drying process would show no increase in virulence (assuming the avirulent mutants remaining in the dried product are capable of multiplying as rapidly as the virulent cells). (b) If the loss in virulence during drying were caused by a physiological change, e.g., damage to or the destruction of a particular enzyme system, then the dried cells would be able to repair the damage during multiplication and thus regain their original virulent state. The latter hypothesis appears to be the correct one. The following experiment supports this conclusion.

(U) A loopful of growth from an agar slant culture of P. pseudomallei was placed in 20 ml of beef extract sorbitol broth. The culture was incubated with aeration for 24 hours at 37°C. Eight 300-ml Erlenmeyer flasks containing 20 ml of broth each were inoculated with 0.2 ml of cell suspension from the above culture. These eight flasks were incubated on a 37°C water-bath shaker for 24 hours. The growth from four flasks (80 ml) was combined and mixed with 80 mg of ingredients of a modified Naylor's preparation (9.10 per cent riboflavin; 45.45 per cent thiourea; and 45.45 per cent ascorbic acid). The growth from the remaining four cultures was also combined. Samples of the two cell suspensions were removed for viable counts and virulence determinations. Four-ml aliquots of the culture containing the modified Naylor's constituents were placed in 10-ml vaccine bottles. The plain broth culture also was dispensed in four-ml amounts into another set of 10-ml vaccine bottles. The material was frozen at 50°C and lyophilized in the freeze-dryer; the dry products were then resuspended to original liquid volume with sterile distilled water. The LD_{50} of the resuspended dry material was determined by injecting 1.0-ml amounts of serial tenfold dilutions, estimated to bracket the anticipated endpoint, into the intraperitoneal cavity of hamsters. A 0.2-ml aliquot of the material dried in the mother broth and reconstituted in distilled water was used to inoculate 20 ml of beef extract sorbitol broth. These flasks were incubated with shaking for 24 hours at 37°C and the viable count and the hamster LD_{50} were determined. Results obtained from four replicate experiments are shown in Table XIX.

(U) An examination of the data in Table XIX reveals that cells dried in the mother broth underwent a two- to four-log drop in viability and an increase in the hamster LD_{50}. The cultures dried in mother broth containing modified Naylor's constituents exhibit a four- to five-log drop in viable count but still retain their original virulence. Cells dried in mother broth regain their original virulent state on subculture in beef extract sorbitol broth. This indicates that the reduction in virulence of the cultures dried in mother broth is a physiological rather than a genetic change. Otherwise, one would not expect the subculture or the cells dried in modified Naylor's solution to be as virulent as the liquid cultures before drying.

TABLE XIX. (U) VIABILITY AND VIRULENCE OF P. PSEUDOMALLEI BEFORE AND AFTER DRYING IN MOTHER BROTH AND IN MOTHER BROTH PLUS CONSTITUENTS OF MODIFIED NAYLOR'S SOLUTION

CULTURE	LD_{50}, cells				VIABLE CELLS/ml			
	Replicate				Replicate			
	1	2	3	4	1	2	3	4
Before Drying in MB[a]/	2.9	<41	1	6	2.0×10^{10}	4.1×10^{10}	2.8×10^{10}	2.9×10^{10}
MB plus modified Naylor's solution	1	1	1.5	9	7.6×10^{9}	2.3×10^{10}	1.5×10^{10}	2.8×10^{10}
After Drying in MB								
Resuspended in distilled water	40	93.5	30	40	$<10^{6}$	2.9×10^{8}	9.3×10^{8}	4.0×10^{6}
Plus modified Naylor's solution and resuspended in distilled water	--	1.10	1.0	9.4	$<10^{6}$	1.0×10^{5}	1.0×10^{4}	1.6×10^{6}
Subcultured in beef extract sorbitol broth	1	< 30	1.5	1	1.7×10^{9}	3.0×10^{10}	1.5×10^{10}	2.0×10^{10}

a. Mother broth.

2. (C) Viability and Virulence After Drying

(C) After the reduction in virulence of P. pseudomallei during lyophilization was shown to be a physiological change, the next step was to investigate various suspending fluids that could be used as drying menstrua and would preserve the virulence as well as increase the percentage of cells surviving the drying process. Cells grown in beef extract sorbitol broth, centrifuged, and suspended in a five per cent sucrose solution showed no apparent loss of virulence for hamsters after lyophilization. Recoveries averaged 10 to 30 per cent of the viable population (Table XX).

(C) The addition of 2.5 per cent skim milk to the sucrose solution increased the average viable recovery after lyophilization to 35 per cent. Results of preliminary experiments indicate that the antioxidants (thiourea and ascorbic acid in 0.5 per cent concentration) could be added to the sucrose skim-milk diluent without adversely affecting virulence (Table XXI).

G. (C) STORAGE STABILITY OF LIQUID SUSPENSIONS OF P. PSEUDOMALLEI

(C) The storage stability of liquid preparations was tested at -70, -20, 4 and 25°C. Cells for the suspensions were obtained from cultures grown in 15-liter amounts of beef extract glycerol broth in five-gallon carboys. The cells were sedimented by centrifugation and then resuspended to the desired concentration in the following test diluents: (a) gelatin saline, (b) spent BES broth plus buffer, and (c) fresh BES broth. One-milliliter samples of the different cell suspensions were dispensed in small polyethylene syrettes and held at each temperature mentioned above. At specified intervals two or more of these syrettes were selected at random from each storage temperature to determine viable cell count and virulence for hamsters. Additional storage experiments were performed in which 1500-ml beef extract sorbitol broth cultures of P. pseudomallei were grown in 2000-ml quantities for varying periods. The results of these studies are shown graphically in Figures 19, 20, 21, 22 and 23. Figure 24 shows the storage stability of suspensions derived from solid media. Although there was a steady decline in viable cell counts with storage time, virulence for the hamster by the intraperitoneal route or by aerosol inhalation did not decline to any appreciable extent. Virulence of products stored for six weeks at 4°C and lower remained unchanged. Storage at 4 to 5°C would apparently be suitable for six to nine weeks if a concentration of 1×10^9 viable cells per milliliter were acceptable. The mother broth is a suitable storage menstruum and no processing of cultures after production is required. However, as noted previously, the aerosol stability decreases with storage.

TABLE XX. (U) VIABILITY AND VIRULENCE OF P. PSEUDOMALLEI LYOPHILIZED IN FIVE PER CENT SUCROSE SOLUTION

CULTURE	LD_{50}, cells			VIABLE CELLS/ml		
	Experiment			Experiment		
	1	2	3	1	2	3
WET						
Beef sorbitol broth	1.2			1.27×10^{10}		
Cells suspended in five per cent sucrose solution	1.0	1.5	2.7	7.7×10^{9}	1.5×10^{9}	2.6×10^{10}
Broth subculture of cells grown and dried in mother broth	1.0			8.0×10^{9}		
DRY						
Cells grown and dried in mother broth	17			5.3×10^{6}		
Cells dried in five per cent sucrose solution	1.0	1.0	5.2	3.1×10^{9}	1.8×10^{8}	2.8×10^{9}

TABLE XXI. (C) EFFECT OF VARIOUS ANTIOXIDANT SOLUTIONS ON BIOLOGICAL RECOVERY AND VIRULENCE OF P. PSEUDOMALLEI AFTER LYOPHILIZATION

DRYING MENSTRUUM		MEAN BIOLOGICAL RECOVERY AFTER LYOPHILIZATION, %	HAMSTER IPLD$_{50}$, cells
SS diluent[a] (control)		35.4[b]	10
Thiourea	1.0%	<1.0	--[c]
	1.0% + SS diluent	21.1	>200
	0.5% + SS diluent	60.1	<10
	1.0% + sucrose, 5%	4.7	10
Ascorbic acid	0.5%	<1.0	--
	0.5% + thiourea 0.5%, + SS diluent	48.1	10
	0.5% + SS diluent	34.6	<10
Citric acid	0.5%	<1.0	--
	0.5% + ascorbic acid 0.5% + SS diluent	<1.0	--
	0.5% + thiourea 0.5%, + ascorbic acid 0.5% + SS diluent	<1.0	--
	0.5% + thiourea 0.5% + SS diluent	<1.0	--
	0.5% + SS diluent	<1.0	--

a. Five per cent sucrose plus 2.5 per cent skim milk.
b. Mean of nine independent trials; all other figures are the mean of two independent trials.
c. No results.

H. (U) THERAPY STUDIES

1. (C) In vitro Sensitivity Tests to Antibiotics

(C) The in vitro sensitivity of P. pseudomallei, Strains 8016, 1454, 1455, 1456, 1457 and 1458, to chlortetracycline, oxytetracycline, tetracycline and chloramphenicol were tested by the disk method[31] and the Szybalski or gradient plate technique.[32]

(U) The disk method was used in tests in which beef extract glycerol agar plates were inoculated with 0.1 ml of a 1:100 dilution of a 24-hour beef glycerol broth culture of each strain of P. pseudomallei; then sterile disks impregnated with antibiotics were placed on the plates. Zones of inhibition were measured and recorded after 24 hours' incubation at 37°C. The results are recorded in Table XXII.

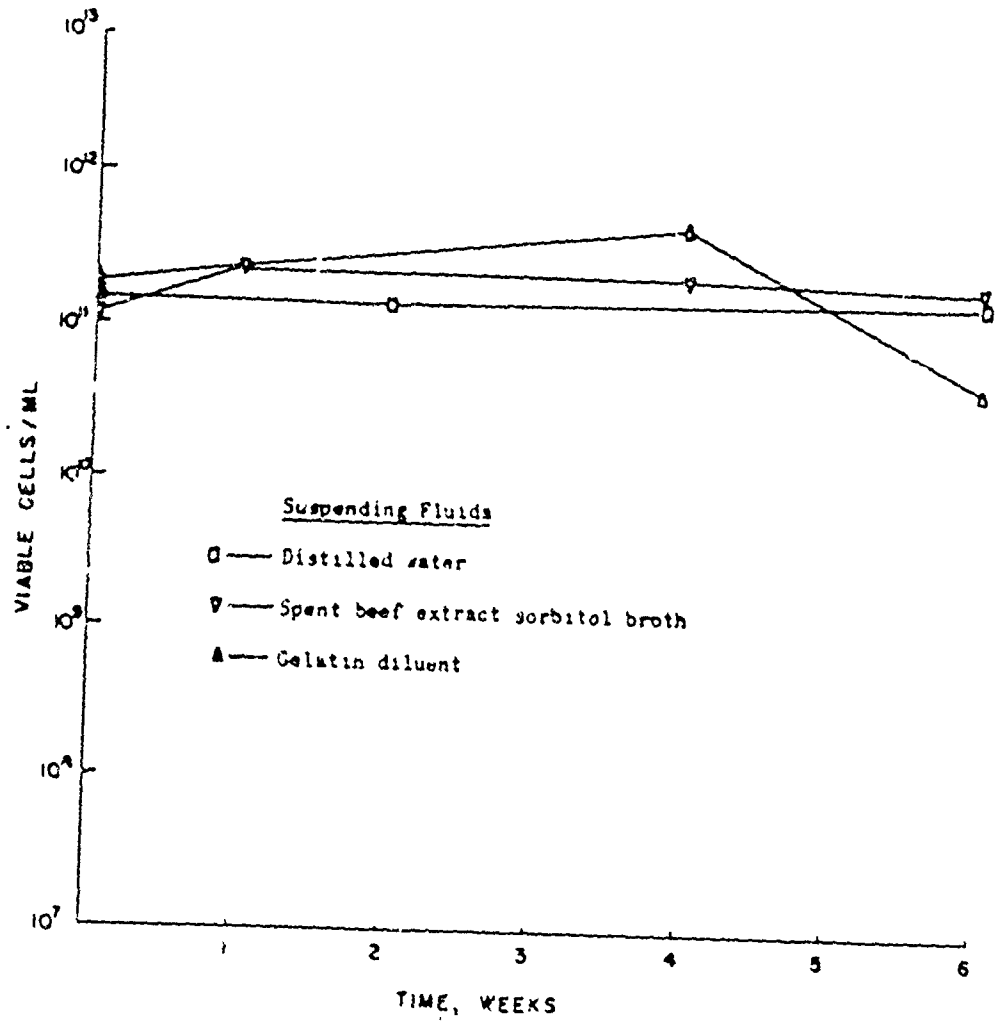

Figure 13. (C) Stability of P. pseudomallei Stored in Various Menstrua at -70°C.

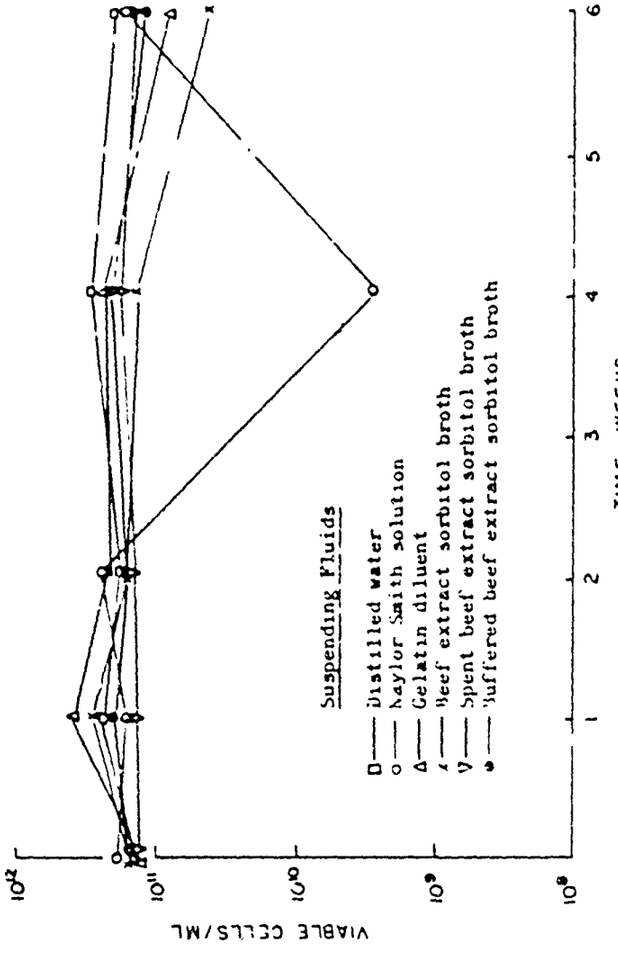

Figure 20. (C) Stability of P. pseudomallei stored in Various Menstrua at -20°C.

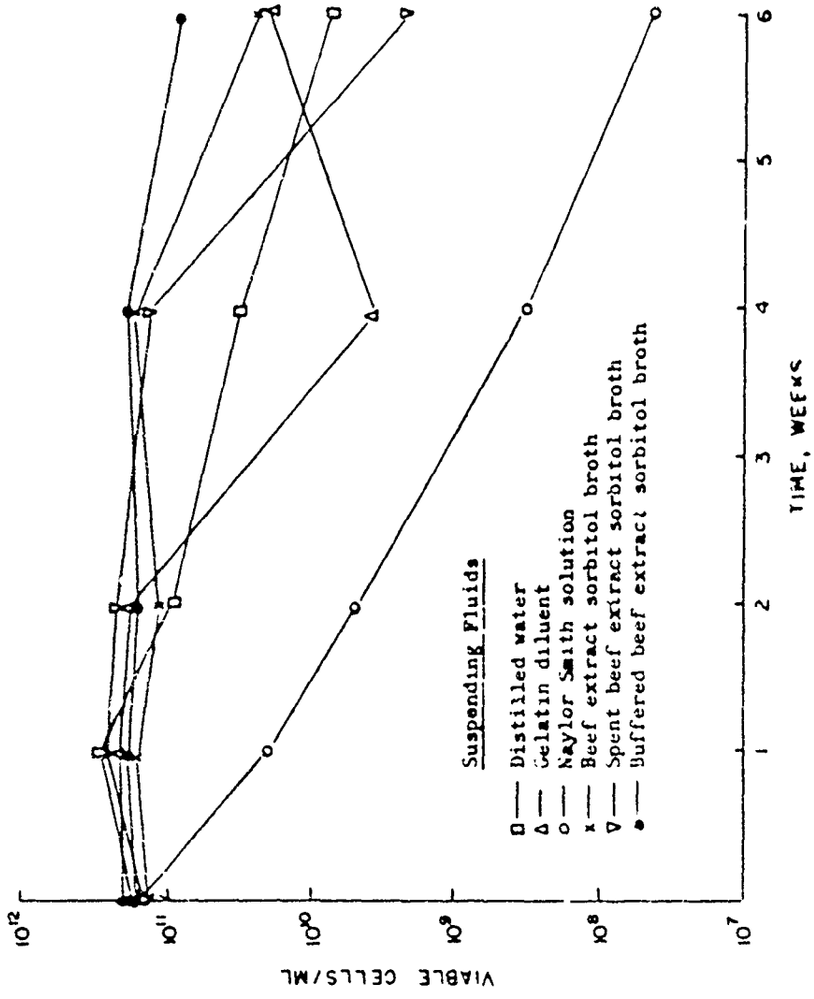

Figure 21. (C) Stability of P. pseudomallei Stored in Various Menstrua at 4°C.

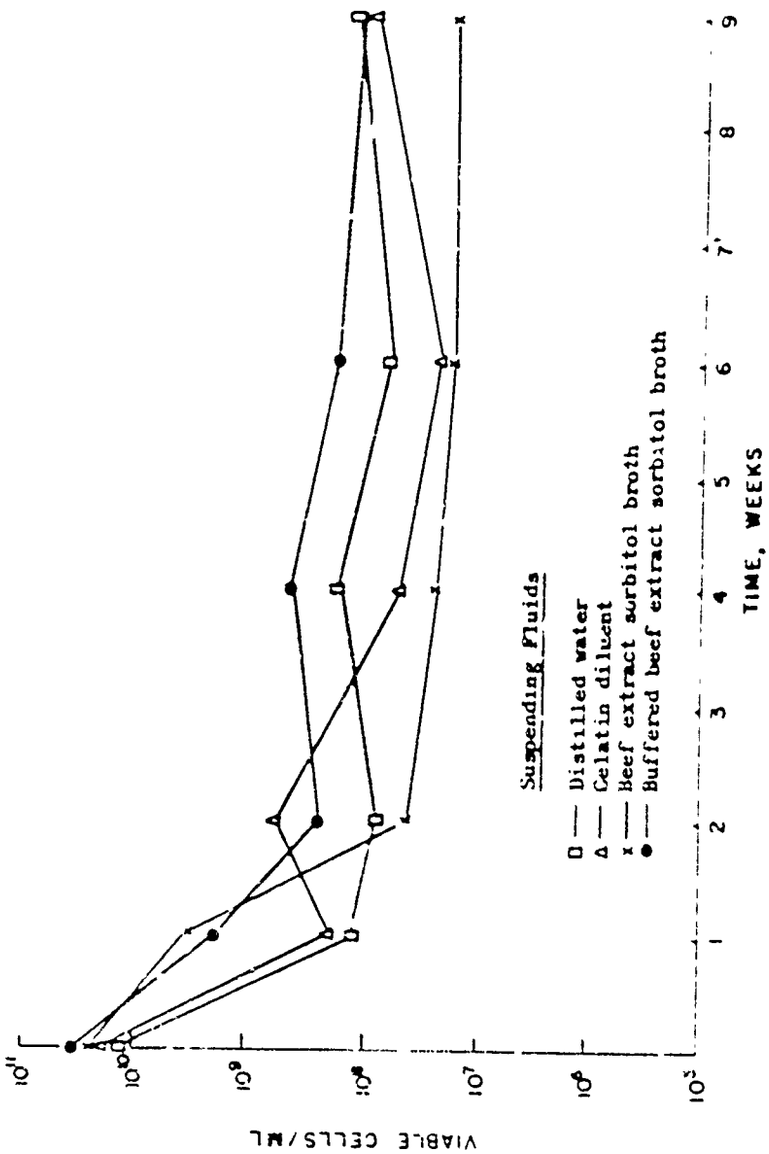

Figure 22. (C) Stability of P. pseudomallei Stored in Various Menstrua at Approximately 25°C.

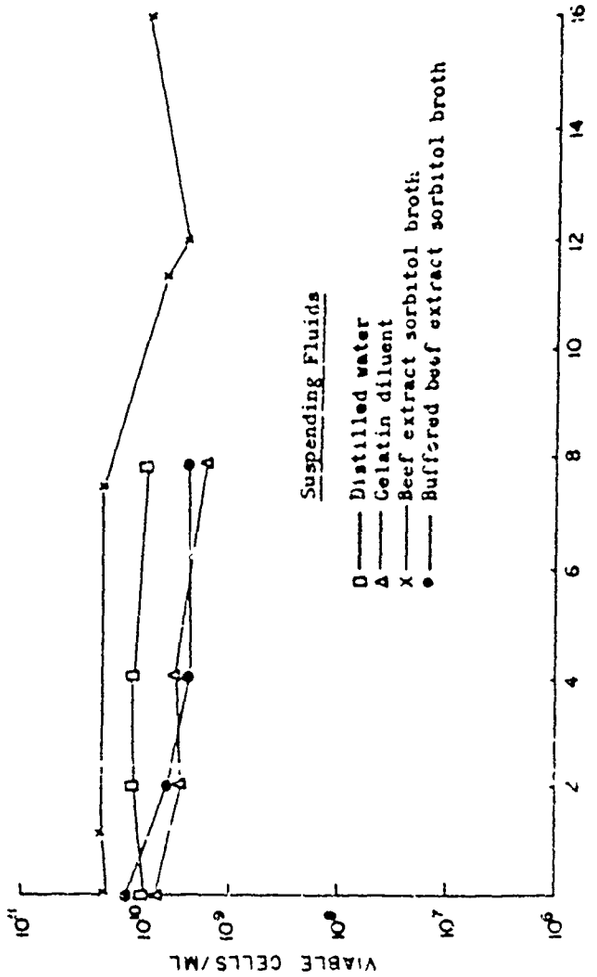

Figure 23. (C) Stability of Various Lots of P. pseudomallei Cultivated in Beef Extract Sorbitol Broth and Stored at 4°C.

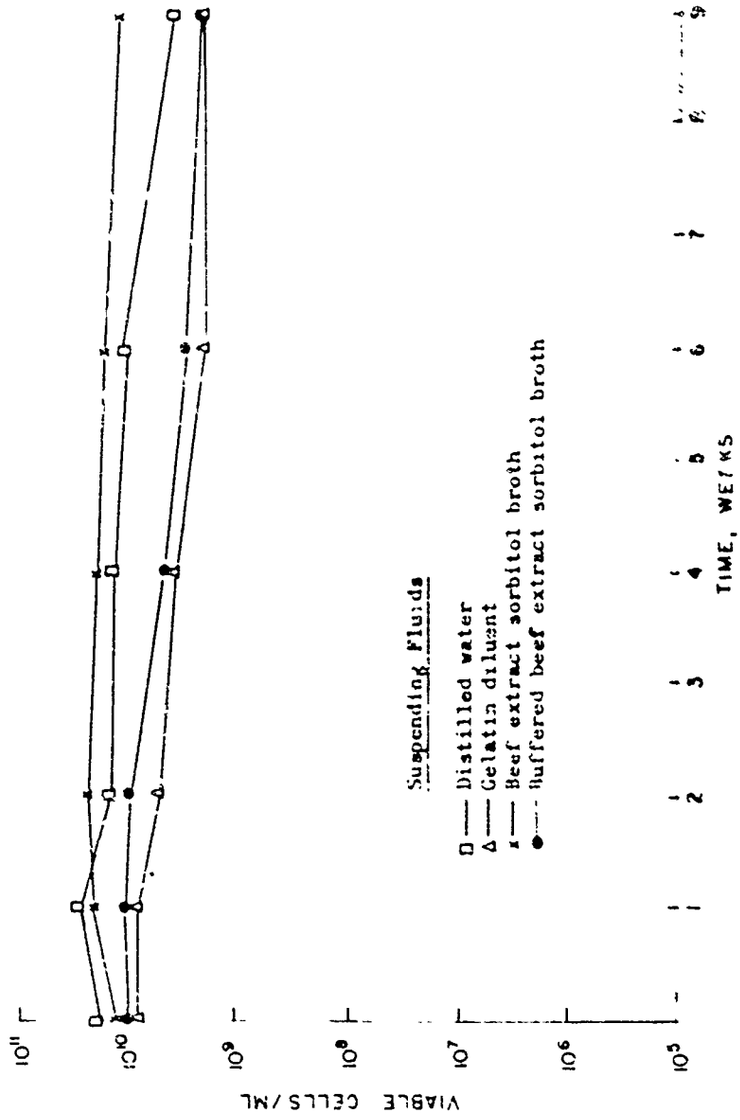

Figure 24. (C) Stability of P. pseudomallei Cells Grown on Beef Extract Glycerol Agar and Stored in Various Menstrua at 4°C.

TABLE XXII. (U) ANTIBIOTIC SENSITIVITY[a] OF SIX STRAINS OF P. PSEUDOMALLEI BY THE DISK METHOD

STRAIN	REPLICATE	ANTIBIOTIC, μg/ml											
		5	10	30	5	10	30	5	10	30	5	10	30
		Chloramphenicol			Chlortetracycline			Oxytetracycline			Tetracycline		
1454	1	0	9	20	20	25	30	0	8	12	9	18	24
	2	0	8	18	19	22	25	0	0	10	12	16	21
	3	8	11	18	17	20	23	0	0	11	15	15	20
1455	1	0	0	0	20	20	25	0	8	13	9	14	20
	2	0	0	0	18	20	24	0	0	12	11	17	22
	3	0	0	0	16	18	20	0	8	12	13	13	18
1456	1	0	8	18	23	23	25	0	0	11	12	17	22
	2	0	12	18	20	20	25	0	0	11	12	16	23
	3	0	13	19	19	19	22	0	0	12	17	18	19
1457	1	0	12	20	20	24	25	0	8	12	14	18	26
	2	8	13	20	19	20	23	0	0	9	12	15	23
	3	0	15	22	15	18	22	0	0	11	17	17	30
1458	1	0	0	0	22	23	29	0	8	12	13	16	25
	2	0	0	0	22	22	24	0	0	10	10	15	23
	3	0	0	0	16	22	24	0	0	12	15	16	22
8016	1	0	0	14	18	23	25	0	0	11	10	15	23
	2	0	0	13	19	21	23	0	0	8	9	19	21
	3	0	0	12	15	19	20	0	0	12	13	14	20

a. Recorded as millimeters of inhibition.

(C) The Szybalski plate technique was used in the following test: 20 ml of beef glycerol agar containing one microgram of antibiotic per milliliter were poured into a sterile Petri dish. The dish was slanted so that the agar just covered the bottom and a gradient of agar thickness was obtained. After the agar hardened, the plate was placed on a flat surface and a second layer of agar, containing 30 micrograms of antibiotic per milliliter, was added to the dish. The plates were put into the refrigerator to allow diffusion of the antibiotic that resulted in a gradient concentration of the test substance which ranged from 1 to 30 micrograms per milliliter. A loopful of a 24-hour beef glycerol broth culture of each of the six P. pseudomallei strains tested was streaked on the agar surface of plates parallel to the concentration-gradient axis. All six strains could be screened for sensitivity to one antibiotic on one gradient plate. The plates were incubated at 37°C and the amount of inhibition of a given antibiotic was determined visually after 24 hours. The results given in Table XXIII are expressed as the approximate concentration of antibiotic in micrograms per milliliter required to inhibit growth.

TABLE XXIII. (C) IN VITRO ANTIBIOTIC SENSITIVITY OF SIX STRAINS OF P. PSEUDOMALLEI

ANTIBIOTIC	P. PSEUDOMALLEI STRAIN					
	1454	1455	1456	1457	1458	8016
Chlortetracycline	1	1	1	1	1	2
Tetracycline	1	5	9	11	11	11
Oxytetracycline	11	11	16	17	17	15
Chloramphenicol	9	30	30	9	30	20

2. (C) Antibiotic Therapy of Experimental Melioidosis in Hamsters

(C) In a preliminary experiment, 2000 micrograms of chlortetracycline, oxytetracycline, or tetracycline given twice daily prolonged the survival time of hamsters infected with 10,000 viable organisms of P. pseudomallei, Strain 8016, but did not prevent death. Chloramphenicol, in the same experiment, proved ineffective. These results suggest that more frequent administration of the first three drugs mentioned above should help maintain a maximum antibiotic concentration in the blood and tissues and might give more favorable results. This possibility was tested in another experiment in which hamsters infected with 1900 viable cells of Strain 8016 were given 2000 micrograms of an antibiotic four times a day.

In addition to the administration of single antibiotics, the effect of 2000 micrograms of tetracycline in combination with either human gamma globulin or human sera was tested on one group of infected animals. Human gamma globulin and human plasma given in conjunction with oxytetracycline or chloramphenicol have protected mice infected with various organisms.[33,34]

(U) Forty-five hamsters each were injected intraperitoneally with 1900 viable cells from a 24-hour-old beef extract sorbitol broth culture of P. pseudomallei, Strain 8016, whose LD_{50} was about one cell. Approximately three hours later antibiotic therapy was initiated. One group of infected animals received one milliliter (2000 micrograms) of antibiotic by the subcutaneous route every six hours; a second lot of five animals receive five micrograms of human gamma globulin (Poliomyelitis Immune Globulin, Lederle Laboratories) plus 2000 micrograms of tetracycline every 12 hours. A third group of infected animals received 0.1 ml of human sera plus 2000 micrograms of tetracycline every 12 hours. The controls consisted of infected animals given no treatment, infected animals receiving five milligrams of human gamma globulin every 12 hours, and uninfected animals injected with antibiotic every six hours. Therapy was administered to surviving animals over a period of four days.

(U) The results of the above experiment, recorded in Table XXIV show that chlortetracycline, oxytetracycline, and tetracycline given in 2000-microgram doses every six hours prolonged the survival time of the infected animals. Chloramphenicol and neomycin sulfate had no effect. Human sera plus tetracycline gave little or no protection to the infected animals at the dosage level used.

(U) In a repeat of the above experiment, two additional combinations, tetracycline plus either ethylenediamine tetracetic acid (EDTA) or cortisone acetate, were also assayed for their protective values. A group of hamsters inoculated with 1400 viable cells (about 1400 LD_{50}'s) of P. pseudomallei were treated with antibiotics alone or in combination with various compounds (Table XXV). The therapeutic agents were administered to the animals every six hours for 7½ days. The animals that died after the fourth day of treatment were autopsied and various tissues were collected for histopathological and bacteriological studies.

(U) Chlortetracycline administered alone or in combination with human gamma globulin or human plasma prolonged the survival time of infected animals until therapy was discontinued (Table XXV). The P. pseudomallei organisms isolated from the tissues of infected animals treated with chlortetracycline were as sensitive to this drug as the original stock culture, indicating that the organisms did not become chlortetracycline-resistant in vivo. At autopsy all infected animals dying after the fourth day had grossly visible lesions of various organs including lungs, liver, kidney, heart, testes and spleen. The liver and spleen were most extensively

TABLE XXIV. (U) EFFECT OF TREATMENT OF EXPERIMENTAL MELIOIDOSIS IN HAMSTERS WITH ANTIBIOTICS ALONE AND IN COMBINATION WITH HUMAN GAMMA GLOBULIN OR SERUM

DAILY DOSE OF ANTIBIOTIC, μg		NUMBER OF HAMSTERS[a] SURVIVING Days After Inoculation 1	2	3	4[b]	5	6	7
VIABLE[c] ORGANISMS INOCULATED - 1900								
Chloramphenicol	8,000	0	0	0	0	0	0	0
Chlortetracycline[d]	8,000	5	5	4	4	4	2	2
Oxytetracycline[d]	8,000	5	5	5	3	1	0	0
Tetracycline[d]	8,000	5	5	4	4	2	0	0
	4,000 + 10,000 Human Gamma Globulin	5	4	4	3	1	1	0
	4,000 + 400 Human Serum	5	3	1	1	0	0	0
Neomycin Sulfate	8,000	1	0	0	0	0	0	0
Human Gamma Globulin	10,000	4	0	0	0	0	0	0
Control	0	1	0	0	0	0	0	0
VIABLE ORGANISMS INOCULATED - NONE[e]								
Chloramphenicol	8,000							
Chlortetracycline	8,000							
Oxytetracycline	8,000	5	5	5	5	5	5	5
Tetracycline	8,000							
Neomycin Sulfate	8,000							

a. Five hamsters used for each treatment.
b. Therapy discontinued at 1800 hours on the fourth day.
c. LD_{50} of culture = one organism.
d. Total dose of chlortetracycline, oxytetracycline, or tetracycline given to survivors was 36,000 micrograms (36 mg).
e. All animals survived.

TABLE XXV. (U) EFFECT OF TREATMENT OF EXPERIMENTAL HELIOIDOSIS IN HAMSTERS WITH VARIOUS ANTIBIOTICS ALONE AND IN COMBINATION WITH COMPOUNDS

ANTIBIOTIC[a] OR COMPOUND Total Daily Dose/Animal, mg	FREQUENCY OF ADMINISTRATION, hr	TOTAL AMOUNT ADMINISTERED AT LONGEST SURVIVAL TIME	NUMBER OF HAMSTERS SURVIVING Days After Inoculation[b] 1 2 3 4 5 6 7 8 9 10 11 12 13
ORGANISMS INJECTED - 1400			
Tetracycline 8,000	6	58 mg	6 6 6 5 5 2 2 1 1 0 0 0 0
8,000 + 20,000 (20 mg) Gamma Globulin	6	58 mg + 145 mg	6 6 6 6 4 1 1 0 0 0 0 0 0
8,000 + 1,000 (0.8 ml) Human Plasma	6	58 mg + .8 ml	6 6 6 4 1 0 0 0 0 0 0 0 0
4,000 + 4,000 (2.0 ml) EDTA[c] (0.5% soln)	12	20 mg + 10 ml	6 6 1 1 0 0 0 0 0 0 0 0 0
4,000 + 800 (0.4 ml) Cortisone	12	16 mg + 3.2 mg	6 2 0 0 0 0 0 0 0 0 0 0 0
4,000	12	20 mg	6 4 3 2 0 0 0 0 0 0 0 0 0
Oxytetracycline 8,000	6	58 mg	6 6 6 6 6 4 1 1 0 0 0 0 0
Chlortetracycline 8,000	6	58 mg	6 6 6 6 5 5 5 5 5 5 5 5 5
8,000 + 20,000 (20 mg Gamma Globulin	6	58 mg + 145 mg	6 6 6 6 6 6 6 6 6 5 4 3 3
8,000 + 1,000 (0.8 ml) Human Plasma	6	58 mg + 5.8 ml	6 6 6 6 5 5 5 5 5 5 5 5 5
Human Plasma 1,000 (0.8 ml)	6	5.0 ml	5 0 0 0 0 0 0 0 0 0 0 0 0
EDTA 0.5% soln) 4,000 (2.0 ml)	12	5.0 ml	2 0 0 0 0 0 0 0 0 0 0 0 0
Cortisone 800 (0.4 ml)	12	1.0 mg	3 1 0 0 0 0 0 0 0 0 0 0 0
Gamma Globulin 20,000 (20 mg)	12	36.0 mg	6 0 0 0 0 0 0 0 0 0 0 0 0
Organism Control 0	0	0	4 0 0 0 0 0 0 0 0 0 0 0 0
ORGANISMS INJECTED - MORE[d]			
Tetracycline 8,000	6	58 mg	
Chlortetracycline 8,000	6	58 mg	5 5 5 5 5 5 5 5 5 5 5 5 5
Oxytetracycline 8,000	6	58 mg	

a. Six hamsters per treatment for the first three antibiotics used alone or in combination, five hamsters for the remainder.
b. Therapy discontinued at 1800 hours of the seventh day.
c. Ethylenediamine tetracetic acid (EDTA) or Cortisone acetate.
d. All animals survived

involved in the infectious process. Microscopically, the multiple lesions of various sizes were characteristic of melioidosis. Giemsa-stained sections of the lesions revealed numerous bacilli within them.

(C) Apparently chlortetracycline is capable of preventing the organism from multiplying in the animal body as long as a certain level of the antibiotic is maintained in the blood and tissues. However, when the chlortetracycline concentration begins to drop, the bacteria probably begin to multiply and kill the host. Thus, it would seem that the normal defensive mechanisms of the hamster are not capable of destroying the bacterial cells while they are under the inhibitive effects of the antibiotics.

(C) The use of EDTA and cortisone in conjunction with tetracycline in this experiment had no apparent protective effect.

3. (C) Treatment of Experimental Melioidosis in Guinea Pigs

(C) In two experiments with guinea pigs, chlortetracycline, tetracycline and oxytetracycline prolonged the survival time of animals infected with large doses (10,500 to 15,000 viable cells) of P. pseudomallei. Chloramphenicol was ineffective (Table XXVI).

(C) A group of guinea pigs were injected intraperitoneally with 10,500 viable cells of P. pseudomallei, Strain 8016. Antibiotic therapy was initiated nine hours after the animals were infected. Infected guinea pigs, with the exception of a control group, were given 3000 micrograms of an antibiotic every six hours for 6½ days followed by 3000 micrograms of an antibiotic given twice daily for five days. Uninfected guinea pigs (antibiotic controls) received the same amount of antibiotics as the infected animals. The animals that died were autopsied and tissues collected for histological and bacteriological examination. This experiment was repeated on a group of guinea pigs injected intraperitoneally with 15,000 organisms per animal. The infected guinea pigs were given 3000 micrograms of an antibiotic every six hours for 6½ days followed by 3000 micrograms given twice daily for an additional eight days.

(C) The results obtained from these two experiments suggest that the guinea pig responds to therapeutic treatment with the tetracyclines more readily than the hamster, probably because of its greater degree of natural resistance.

(C) The microscopic study of tissues of treated and untreated animals did not demonstrate a significant difference among groups. The extent of tissue involvement in the infectious process varied considerably from animal to animal irrespective of the treatment. As noted previously, in certain animals the pathological process did not appear to have been sufficiently extensive to cause death, except perhaps on the basis of a toxemia.

TABLE XXVI. (U) EFFECT OF TREATMENT OF EXPERIMENTAL MELIOIDOSIS IN GUINEA PIGS WITH ANTIBIOTICS

ANTIBIOTIC	TOTAL AMOUNT OF DRUG ADMINISTERED AT LONGEST SURVIVAL TIME, mg	ORGANISMS INJECTED, No.	GUINEA PIGS TREATED, No.	NUMBER OF GUINEA PIGS SURVIVING Days 6	12[a]	14	24	36	45	SURVIVAL TIME FOR 50% OF ANIMALS, Days
EXPERIMENT I.										
Chlortetracycline	108	10,500	6	6	6		3		1	22
Tetracycline	108	10,500	6	6	5		4		1	25
Oxytetracycline	108	10,500	6	6	6		5		2	31
Chloramphenicol	108	10,500	6	5	0		0		0	9
Control for each antibiotic	108	0	3	3	3		3		3	>45
Control	0	10,500	6	5	2		2		1	8.5
EXPERIMENT II.										
Chlortetracycline	132	15,000	12	12		8		5		22
Tetracycline	132	15,000	12	12		9		3		23
Oxytetracycline	132	15,000	12	12		11		9		--[b]
Chloramphenicol	132	15,000	12	12		6		1		12
Controls-										
Chlortetracycline	132	0	3	3		3		3		>36
Tetracycline	132	0	3	3		3		3		>36
Oxytetracycline	132	0	3	3		3		2[c]		>36
Chloramphenicol	132	0	3	3		3		1[d]		23.5
Control	0	15,000	12	11		4		0		13.5

a. Therapy was discontinued twelve days after infection (Experiment I).
b. No results.
c. Dead animal showed no signs of infection with P. pseudomallei.
d. Dead animals showed evidence of infection with P. pseudomallei. They probably became infected by cross-contamination during therapy.

VI. (C) DISCUSSION AND CONCLUSIONS

(C) The findings of Miller and coworkers, Canadian BW research personnel, the Naval Biological Laboratories, and the BW Labs support the conclusion that both Actinobacillus mallei and Pseudomonas pseudomallei possess many of the qualities desired for BW purposes.

(C) The histories of natural infections caused by the glanders and melioidosis organisms indicate that each has a marked biological effectiveness against man. Available epidemiological data suggest, however, that the former is more infective than the latter. In clinically recognized cases melioidosis appears to be more acute and more rapidly fatal than glanders. The high mortality rates recorded in both diseases offer strong supporting evidence for classifying these bacteria as lethal agents. The reported severity of natural infections and the experimental data showing effective doses in animals by oral and other routes of administration indicate a formidable casualty potential for these disease agents in biological warfare. Furthermore, there is no recognized immunization against either glanders or melioidosis.

(C) Actinobacillus mallei is characterized by (a) ease of production and quantitation, (b) high infectivity, and (c) a high fatality rate in experimental animals. The two shortcomings of this bacterium from the Biological Warfare standpoint are a gradual or abrupt loss of virulence and poor stability of stored suspensions. Basic research on the genetic aspects of virulence seems warranted because of its history of high infectivity among laboratory personnel.

(C) The data reported on Pseudomonas pseudomallei suggest that it has suitable properties as a BW agent. The organism can be grown and the concentration can be determined in a few days without complex facilities or media. Storage and aerosol stabilities compare favorably with those of most vegetative bacteria. Liquid suspensions of most of the strains studied maintained virulence in tests with the hamster and guinea pig. Additional research would be necessary to develop information on two important questions: (a) How susceptible is the average person to infection with P. pseudomallei? (b) What is the relationship, if any, between virulence for susceptible laboratory rodents and for man?

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* Symbol omitted from title.